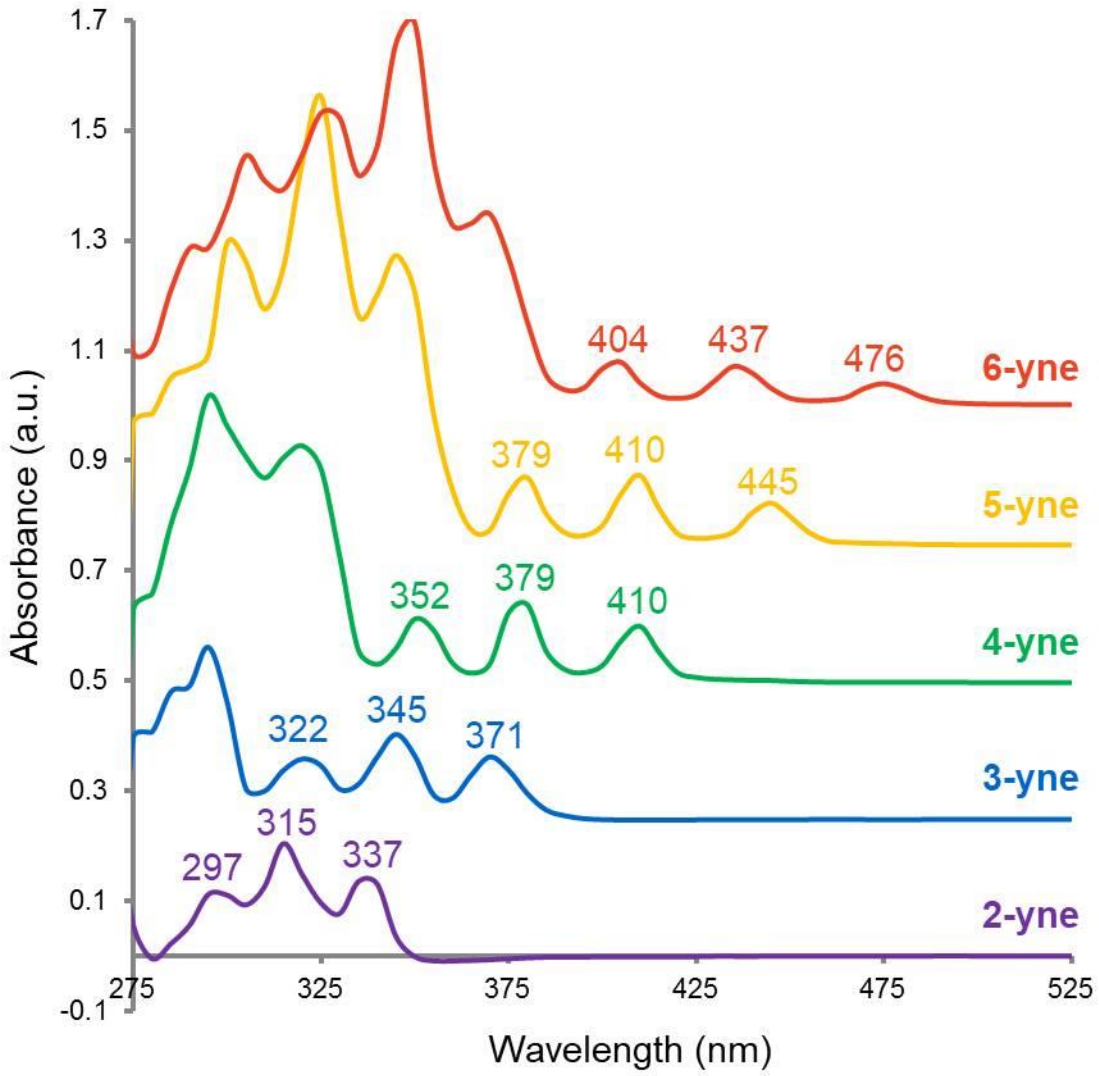
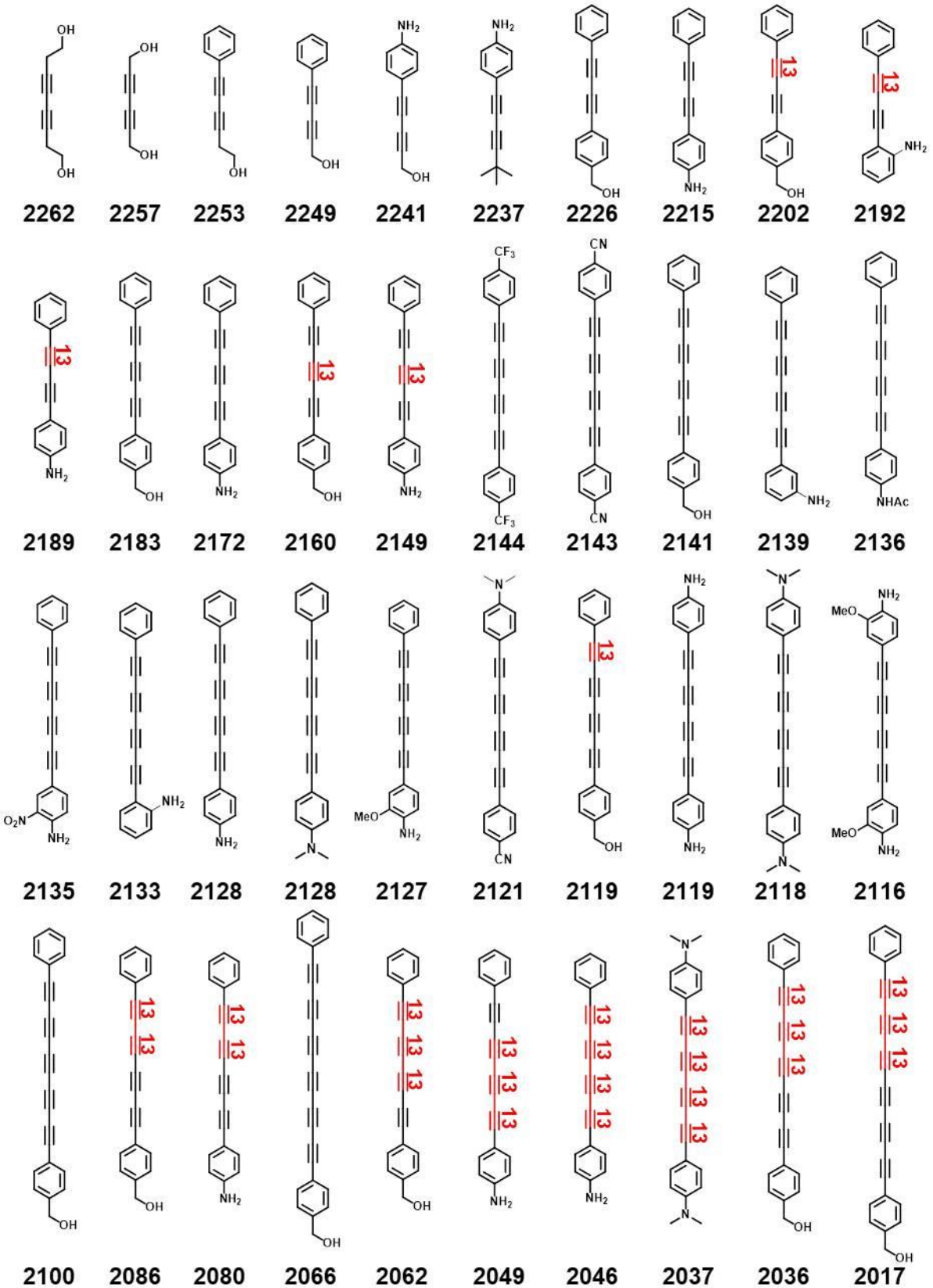


Supplementary Figure 1

UV-Vis absorption spectra of polyynes from 2-yne to 6-yne

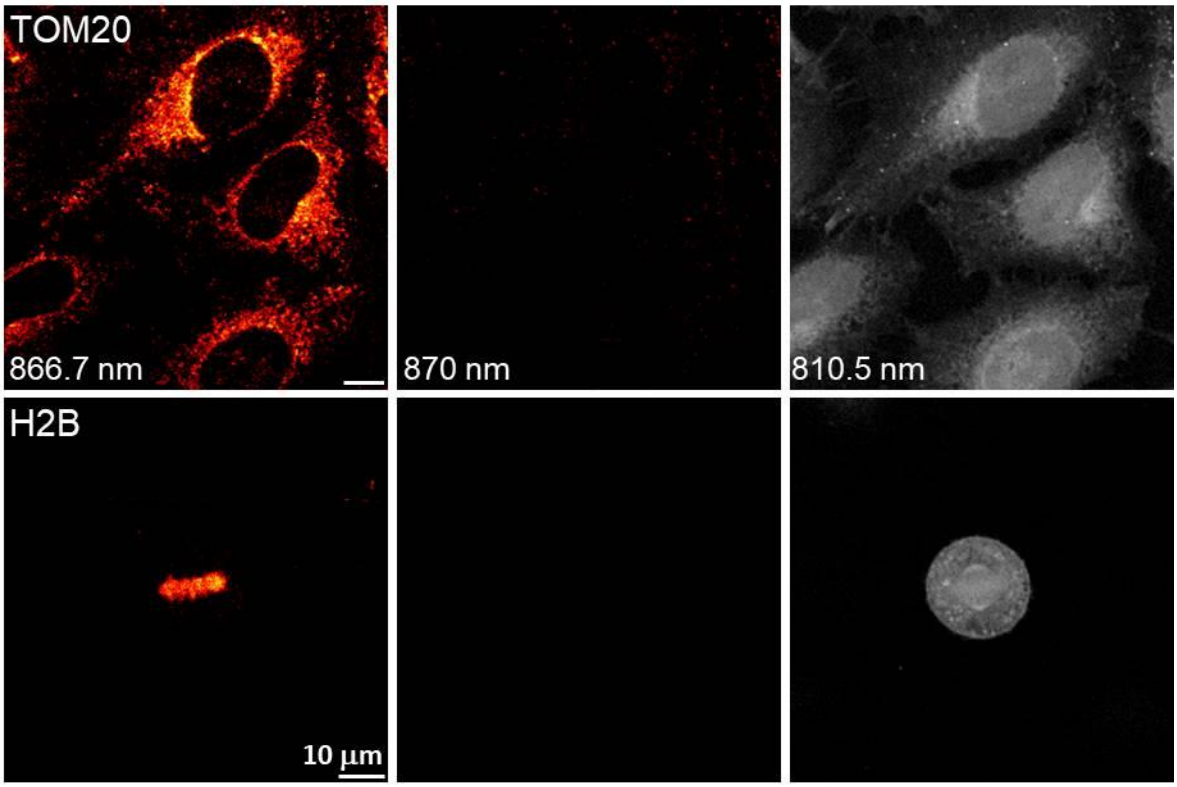
The spectra are vertically offset for clarity. The longest wavelength of absorption red shifts ~35 nm with every additional triple bond. Three sets of peaks are observed at the highest wavelengths as vibrational fine structures, indicating strong vibronic coupling.



Supplementary Figure 2

Frequency exploration of polyynes through conjugation elongation, end-capping variations and isotope doping

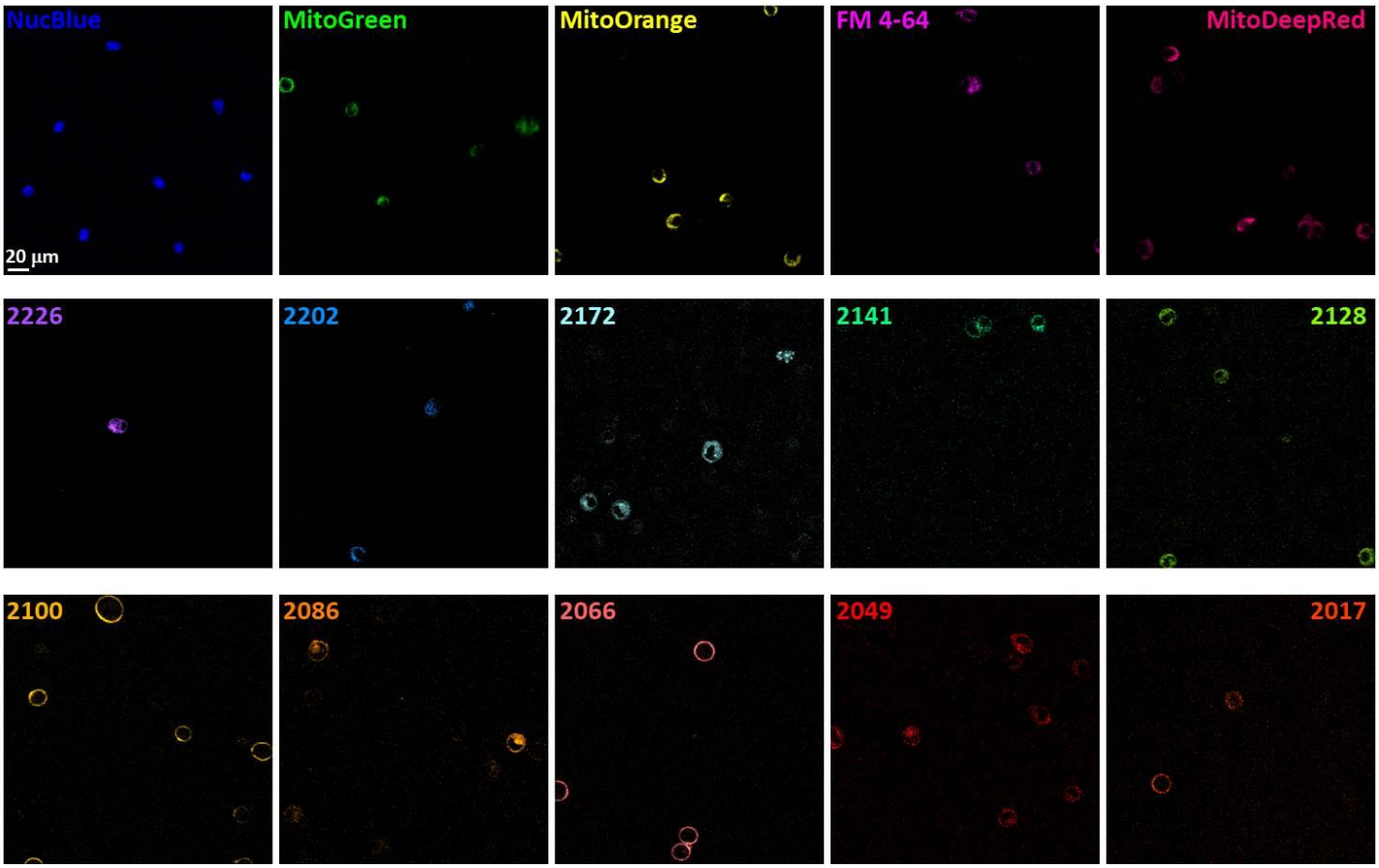
40 structures are synthesized and shown with Raman frequencies (in cm^{-1}) measured in DMSO.



Supplementary Figure 3

Immuno-staining and SRS imaging of TOM20 (mitochondrial marker) and histone H2B (metaphase) in fixed HeLa cells with 4-yne conjugated antibodies

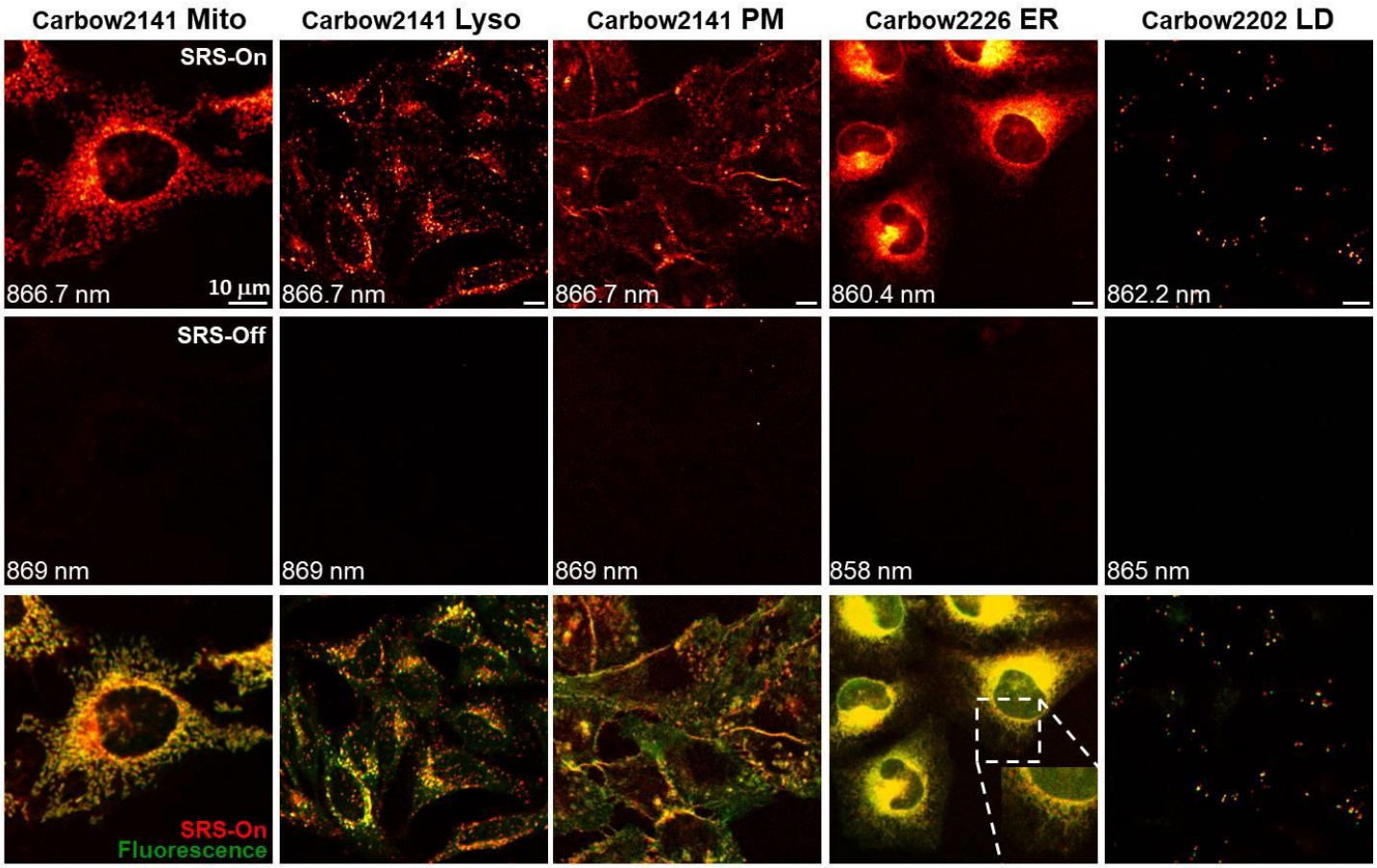
810.5 nm channels show the total protein distribution and outline the cell morphology. Experiments were repeated twice independently with similar results.



Supplementary Figure 4

15-color imaging of live HeLa cells with super-multiplexed polyynes

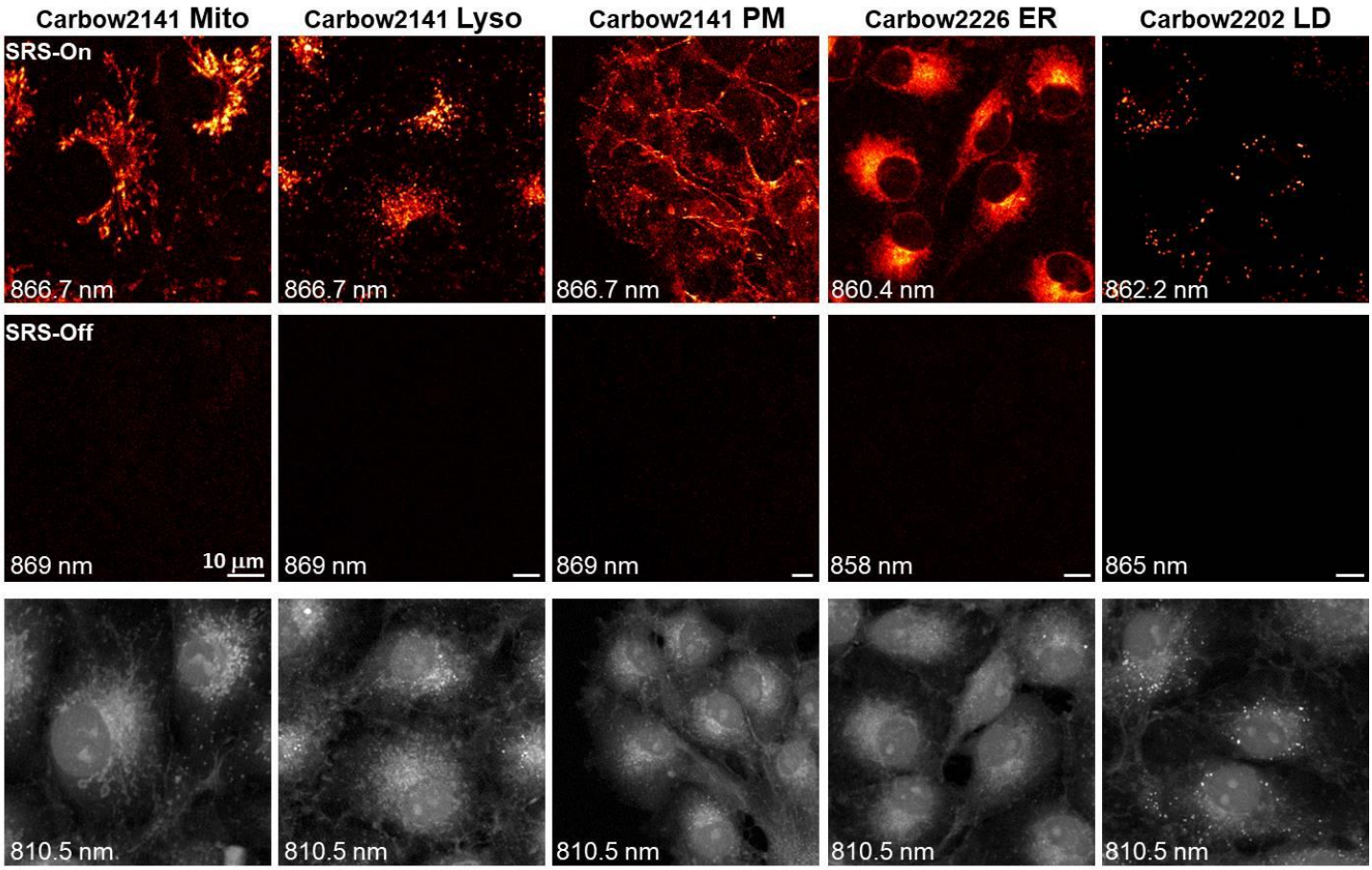
Individual channels of 5 fluorescent dyes and 10 polyynes with well-resolved frequencies are shown with little crosstalk. Unmixing is performed by subtracting the adjacent channel, without the need for matrix unmixing. Experiments were repeated three times independently with similar results.



Supplementary Figure 5

Live-cell SRS imaging of organelle-targeted polyynes and co-localization with fluorescent organelle markers in HeLa cells

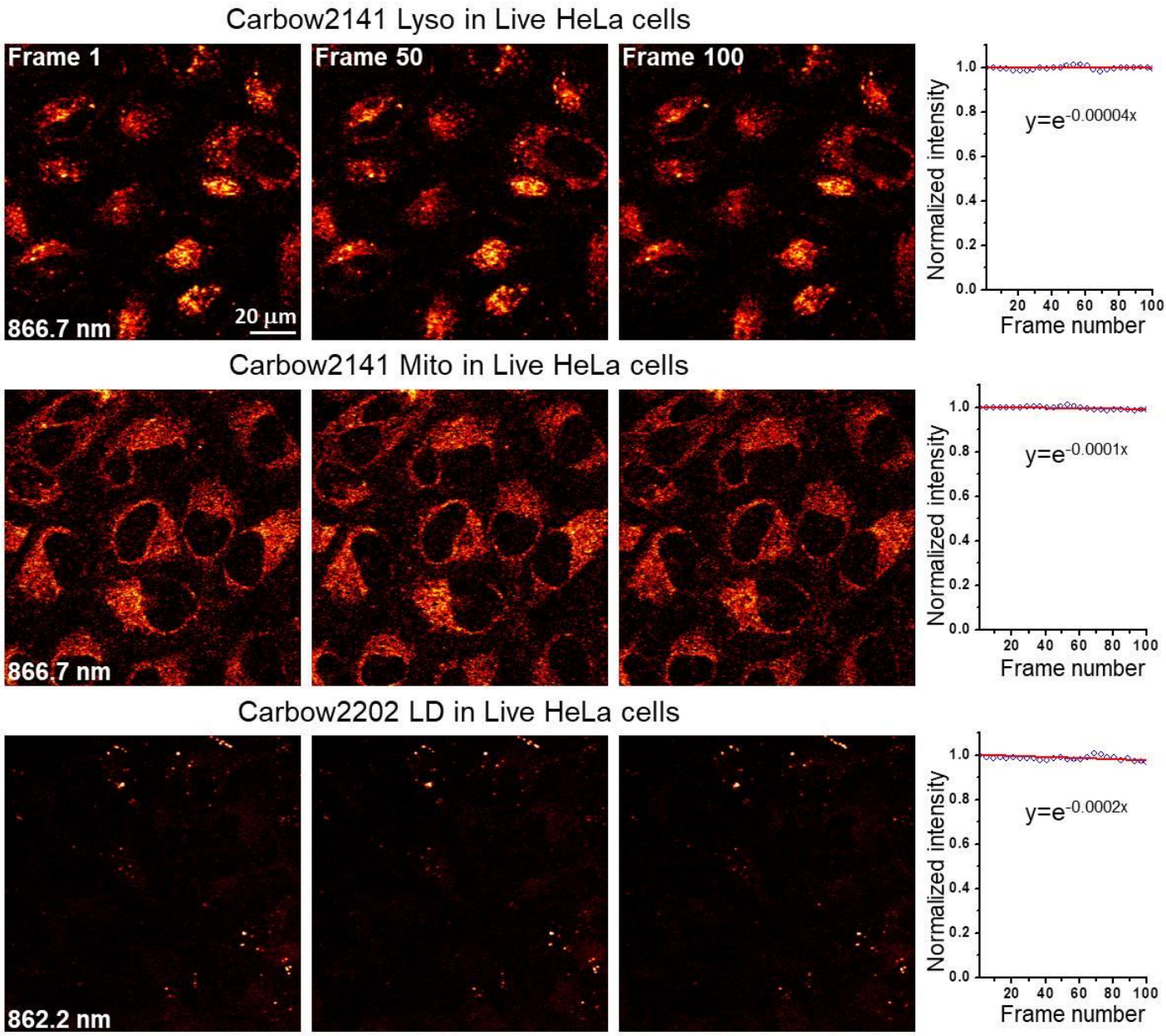
Characteristic labeling patterns are shown for each organelle with negligible crosstalk at 3 nm away. High co-localizations are observed between organelle-targeted polyynes and commercial fluorescent markers. Experiments were repeated five times independently with similar results.



Supplementary Figure 6

Live-cell SRS imaging of organelle-targeted polyynes in COS-7 cells

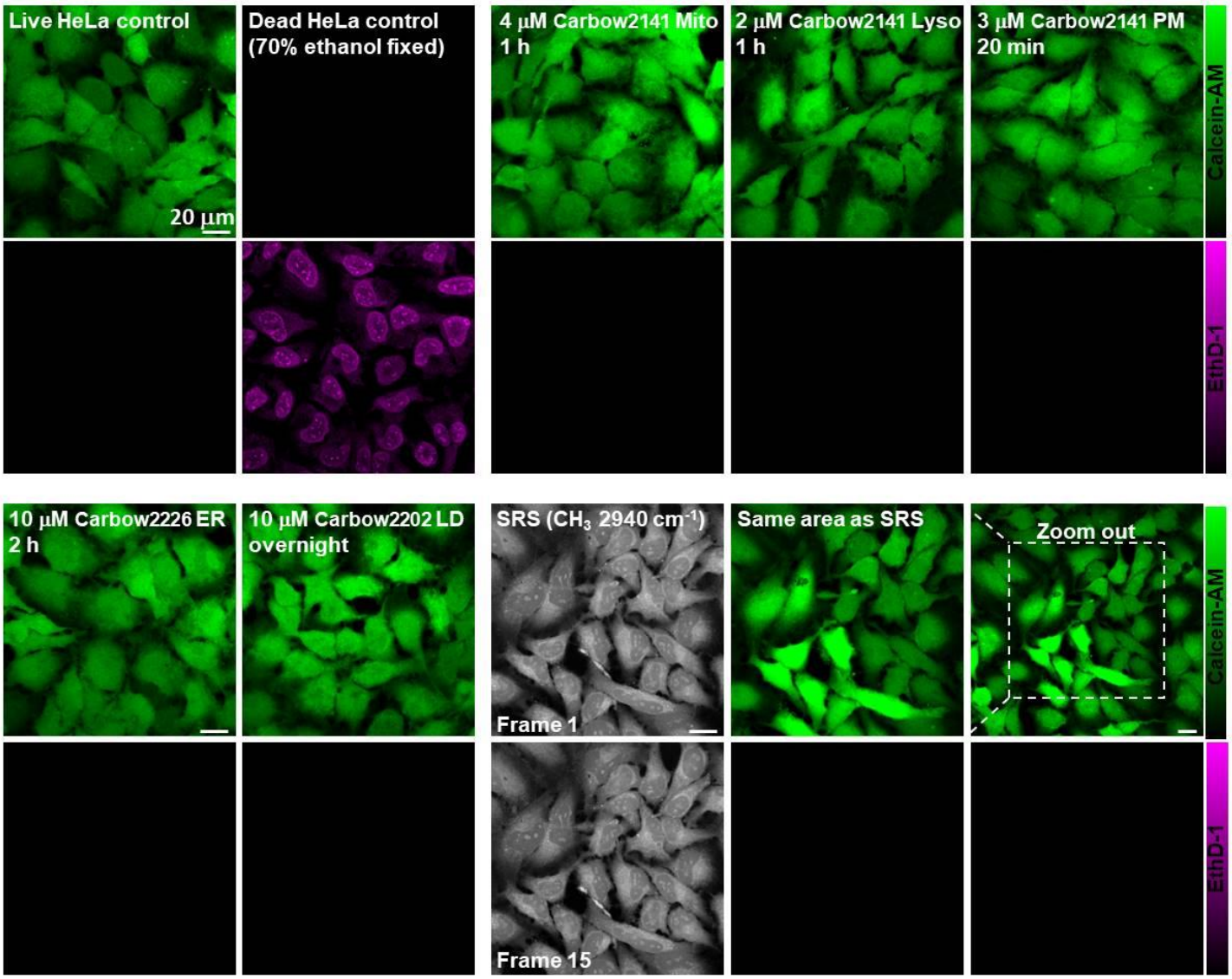
810.5 nm channels show the total protein distribution and outline the cell morphology. Experiments were repeated three times independently with similar results.



Supplementary Figure 7

Excellent photo-stability of polyynes in live cell imaging

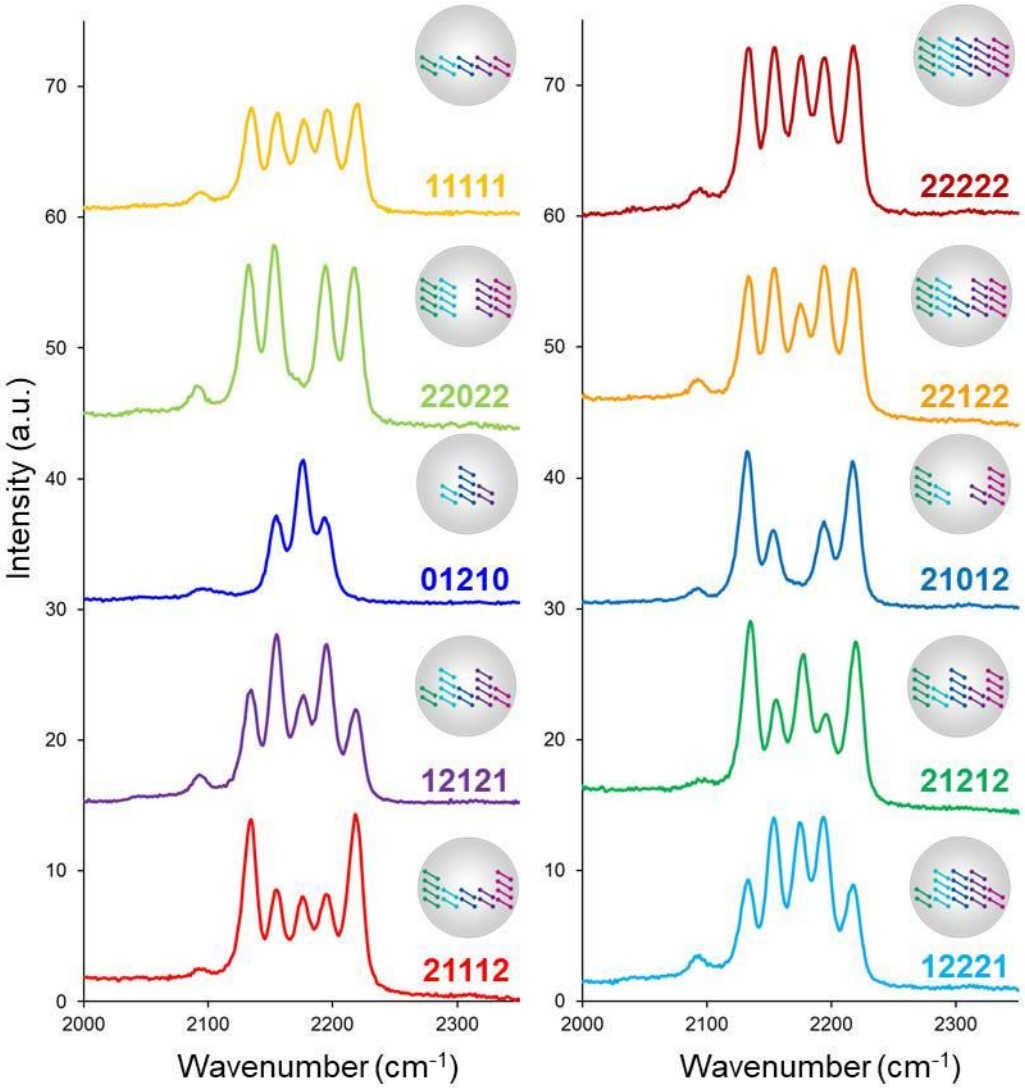
HeLa cells are incubated with 2 μM Carbow2141 Lyso for 1 h, 4 μM Carbow2141 Mito for 1 h or 10 μM Carbow2202 LD for overnight. Cells are continuously imaged for 100 frames with nearly identical intensity, and the intensity trace shows minimal decay (<2%). Experiments were repeated twice independently with similar results.



Supplementary Figure 8

Minimal cytotoxicity of polyynes in live cells and phototoxicity of SRS lasers

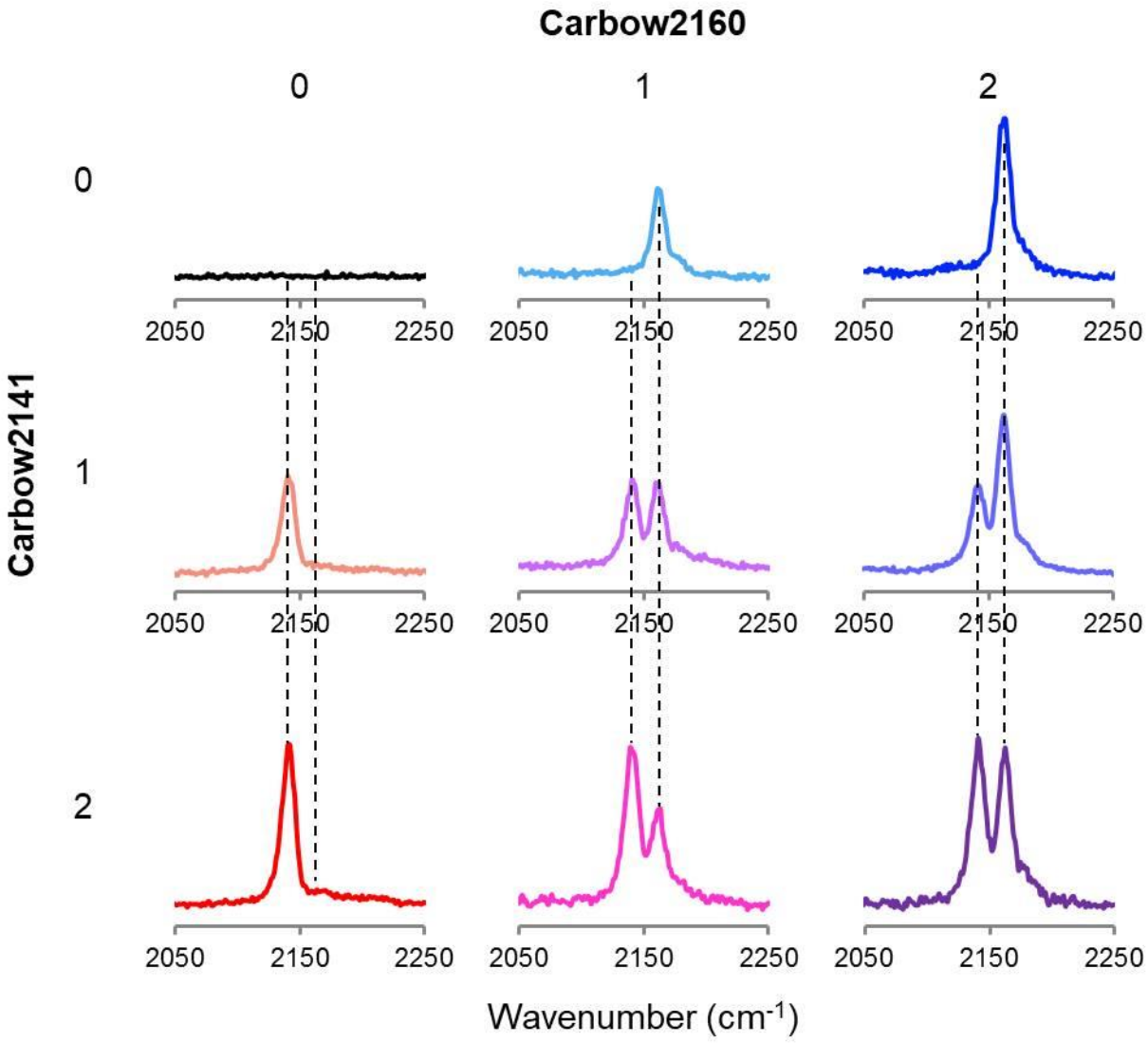
Live and dead HeLa cell standards are verified with Live/Dead viability kit. All five organelle-targeted polyynes exhibit little cytotoxicity in live cells, as shown by two-color imaging of Calcein-AM (green, live-cell marker) and EthD-1 (magenta, dead-cell marker). Also, Minimal phototoxicity is observed in cells with SRS illumination. After 15 frames of continuous SRS imaging at 2940 cm^{-1} (protein CH_3) using the same laser power and dwell time in multiplexed live-cell imaging, the same region of cells show no observable cell death in the viability assays, compared to surrounding cells without SRS laser exposure. Experiments were repeated twice independently with similar results.



Supplementary Figure 9

10 representative spectral barcodes in polystyrene beads by spontaneous Raman microscope

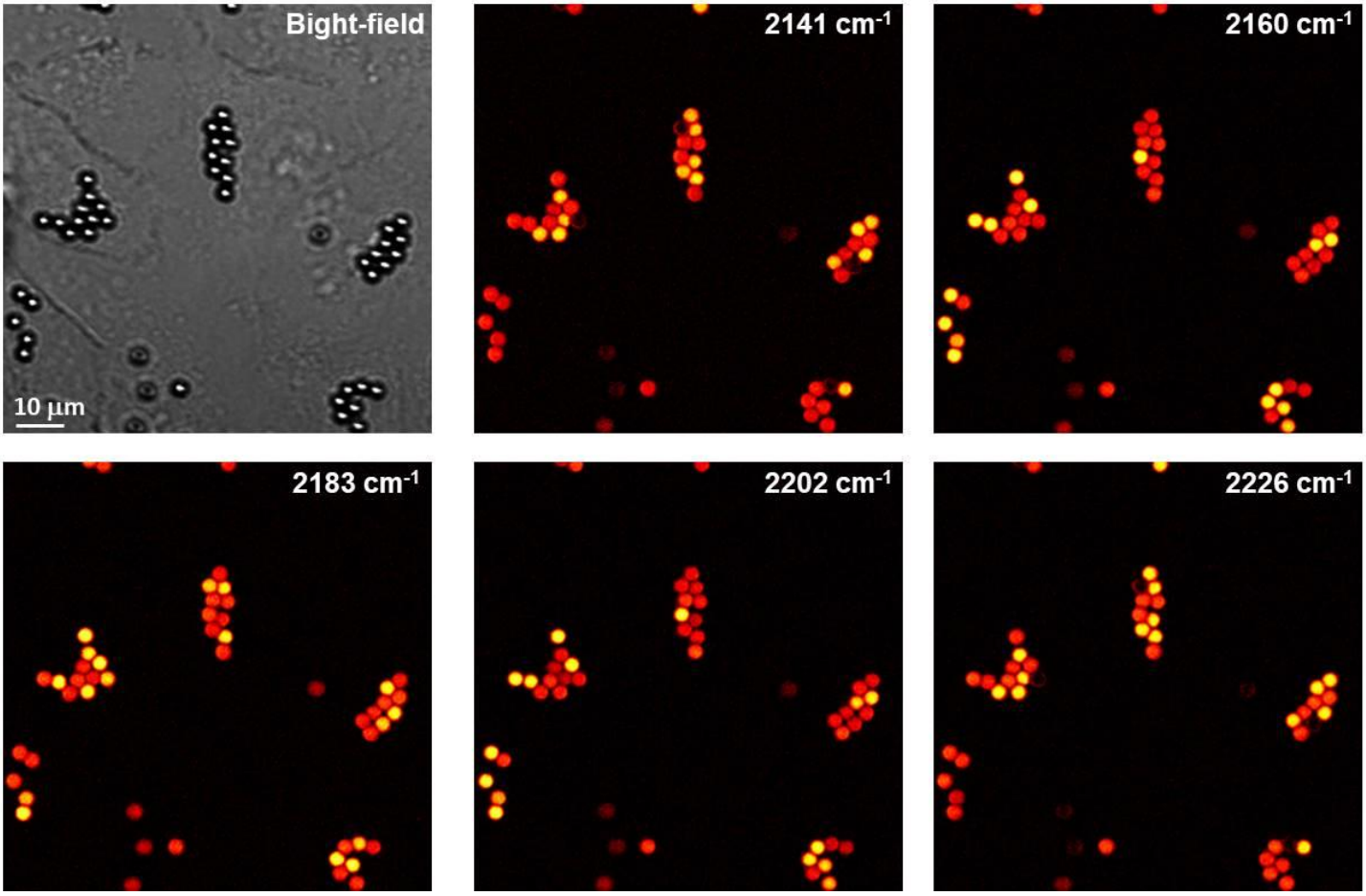
The spectra are vertically offset for clarity. 5 polyynes (Carbow2141, Carbow2160, Carbow2183, Carbow2202 and Carbow2226) that are compatible with 532 nm excitation are used in spectral encoding (Supplementary Table 3) for spontaneous Raman measurement.



Supplementary Figure 10

2-D matrix cross-verification of spectral barcoding with polyynes

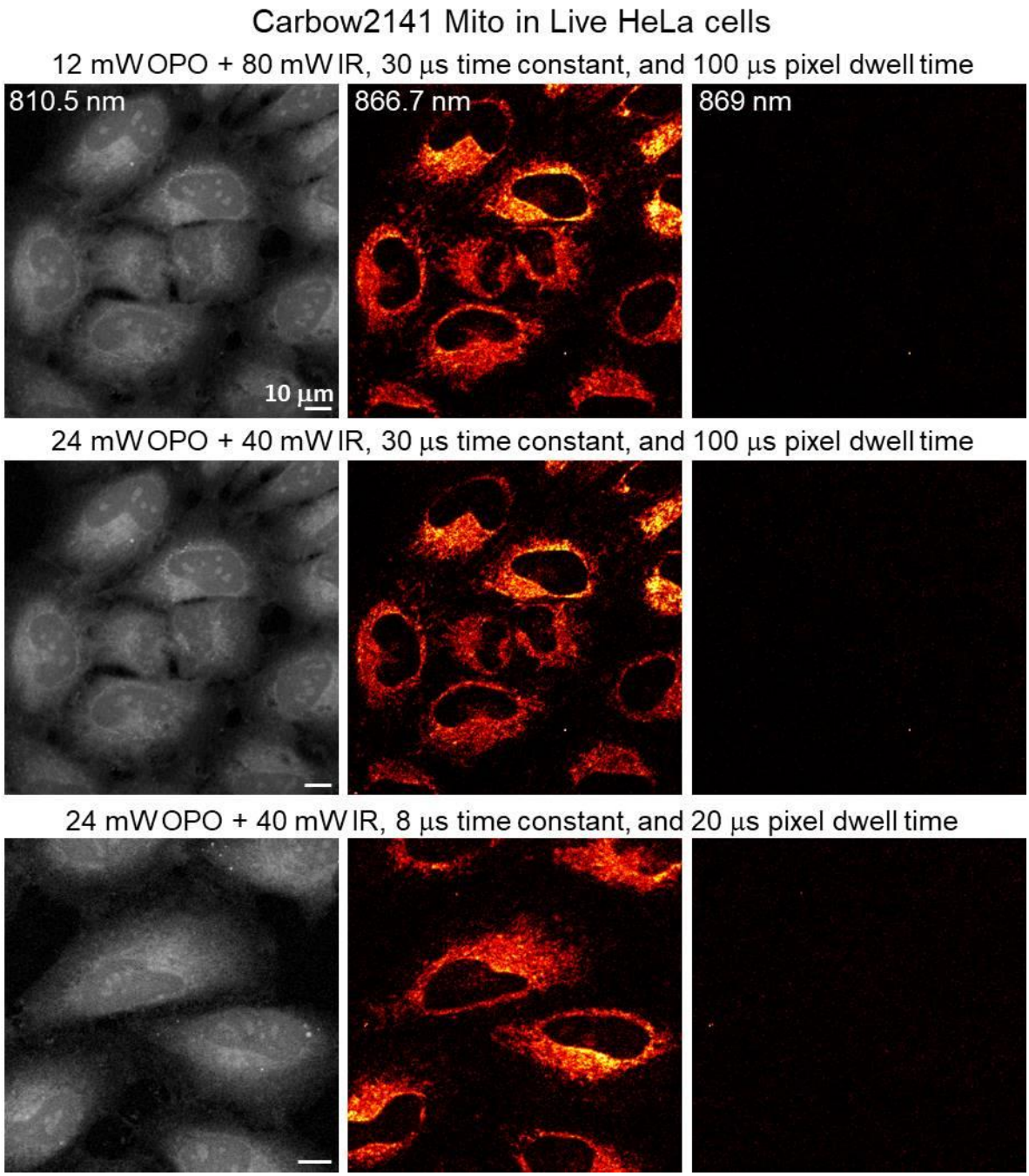
All 9 combinations (3 × 3) of two neighboring frequencies (Carbow2141 and Carbow2160) show well-resolved spectral patterns with little cross-talk, demonstrating the robustness of spectral barcoding.



Supplementary Figure 11

Hyperspectral SRS imaging of encoded beads in live cells

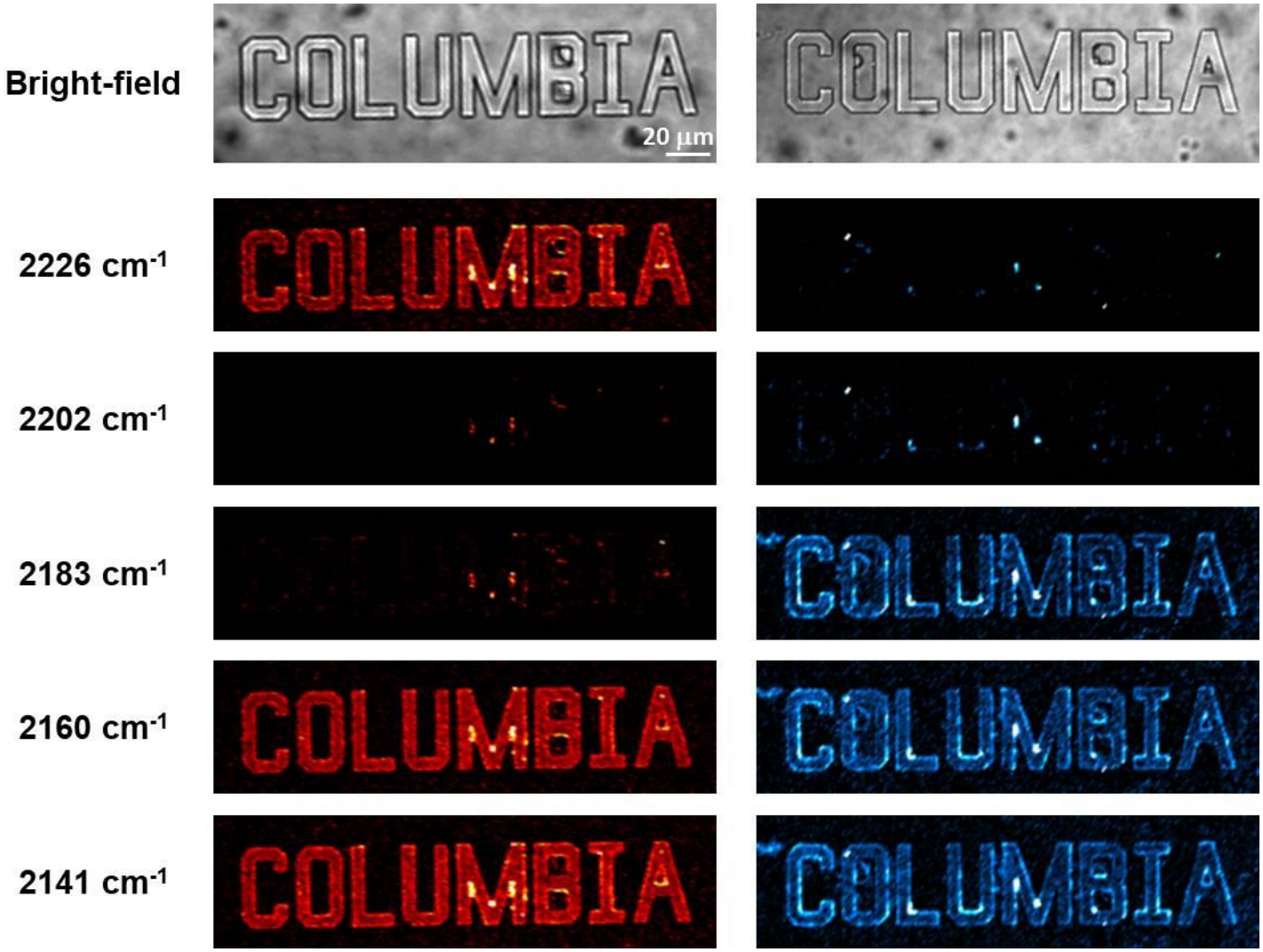
Bright-field image shows the spatial distribution of unidentified beads in cells. Consecutive SRS imaging at characteristic frequencies of polyynes allows rapid decoding and visualization of bead identity in space. Experiments were repeated three times independently with similar results.



Supplementary Figure 12

Fast organelle imaging in live cells with reduced laser power and shorter pixel dwell time

HeLa cells are incubated with 4 μM Carbow2141 Mito for 1 h before imaging. SRS imaging of mitochondria is achieved in as short as 2 seconds per frame. Experiments were repeated twice independently with similar results.



Supplementary Figure 13

Frequency encryption for identity security and anti-counterfeiting with polyynes

Microscopic Columbia logos on PDMS look similar in the bright-field images and hyperspectral SRS images reveal the different identities in the frequency domain.

Supplementary Note

General Information

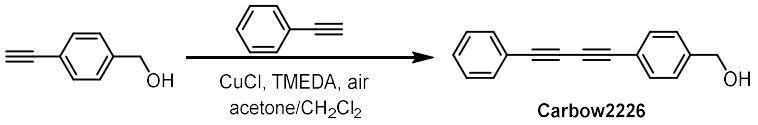
All reagents and solvents were purchased from Sigma-Aldrich and Fisher Scientific and were used without further purification, unless otherwise stated. Flash chromatography was performed on silica gel (Silicycle, 40-63 μm). TLC was performed on 5 mm E. Merck silica plates (60F-254) and visualized by UV light or potassium permanganate ($KMnO_4$) or ceric ammonium molybdate (CAM) stain.

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 500 (500MHz) or Bruker 400 (400 MHz) Fourier Transform (FT) NMR spectrometers at Columbia University, Chemistry Department. NMR spectra were calibrated using residual undeuterated solvent (^{1}H: δ 7.26 for $CDCl_3$, δ 3.31 for MeOH-d_4, δ 2.50 for DMSO-d_6; ^{13}C: δ 77.16 for $CDCl_3$, δ 49.0 for MeOH-d_4, δ 39.50 for DMSO-d_6). The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. High resolution mass spectra (HRMS) were recorded on a XEVO G2-XS Waters mass spectrometer equipped with a QTOF detector with multiple inlet and ionization capabilities. UV-Vis absorption spectra were measured on a Tecan infinite 200 using 96-well plates.

The synthesis of phenyl end-capped polyynes was previously reported by Tykwinski[1,2] and Wulff[3]. In our work, we prepared the Carbow diynes and tetraynes by Glaser-Hay coupling, and the Carbow triynes, pentaynes and hexaynes were synthesized by Cadiot-Chodkiewicz coupling.

Synthesis of Carbow diynes

Carbow2226

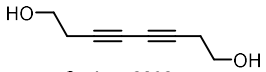


A solution of CuCl (100 mg, 1.0 mmol) and TMEDA (300 μL, 2.0 mmol) in acetone (5 ml) was bubbled with air for 10 mins at rt, then a mixture of 4-ethynylbenzyl alcohol (264 mg, 2.0 mmol) and phenylacetylene (0.66 ml, 6.0 mmol) in CH_2Cl_2 (3 ml) were added and continued to stir with air at rt for 2 h. The solvent was evaporated at reduced pressure and the residue was subjected to chromatography to obtain **Carbow2226** (312 mg, 67%) as a white solid.

^{1}H NMR (400 MHz; $CDCl_3$): δ 7.55-7.50 (m, 4H), 7.40-7.30 (m, 5H), 4.69 (s, 2H), 1.92 (br, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 142.2, 132.8, 132.6, 129.3, 128.6, 126.9, 121.9, 121.0, 81.7, 81.5, 74.1, 74.0, 64.9; HRMS (ASAP): calcd for $C_{17}H_{12}O^+$ $[M]^+$ 232.0888, found 232.0889.

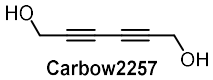
Nature Methods: doi:10.1038/nmeth.4578

The other **Carbow** diynes were prepared using the same procedures as **Carbow2226**.



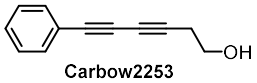
Carbow2262

^{1}H NMR (400 MHz; $CDCl_3$): δ 3.78-3.68 (m, 4H), 2.52 (t, J = 6.2 Hz, 4H), 2.17 (br, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 74.8, 66.9, 60.9, 23.7; HRMS (ASAP): calcd for $C_8H_{11}O_2^+$ $[M+H]^+$ 139.0759, found 139.0760.



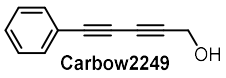
Carbow2257

^{1}H NMR (400 MHz; MeOH-d_4): δ 4.19 (s, 4H); ^{13}C NMR (100 MHz, MeOH-d_4): δ 78.8, 69.4, 50.9; HRMS (ASAP): calcd for $C_{12}H_{12}O_4^+$ $[2M]^+$ 220.0736, found 220.0726.



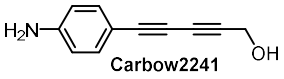
Carbow2253

^{1}H NMR (400 MHz; $CDCl_3$): δ 7.50-7.45 (m, 2H), 7.37-7.27 (m, 3H), 3.84-3.75 (m, 2H), 2.64 (t, J = 6.2 Hz, 2H), 1.96 (br, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 132.7, 129.2, 128.5, 121.8, 81.1, 75.5, 74.1, 66.9, 60.9, 24.1; HRMS (ASAP): calcd for $C_{12}H_{11}O^+$ $[M+H]^+$ 171.0810, found 171.0805.



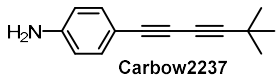
Carbow2249

^{1}H NMR (400 MHz; $CDCl_3$): δ 7.52-7.46 (m, 2H), 7.39-7.28 (m, 3H), 4.43 (s, 2H), 2.28 (br, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 132.7, 129.4, 128.5, 121.5, 80.6, 78.7, 73.3, 70.5, 51.7; HRMS (ASAP): calcd for $C_{11}H_7^+$ $[M-OH]^+$ 139.0548, found 139.0565.



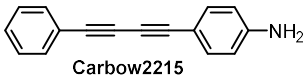
Carbow2241

^{1}H NMR (400 MHz, DMSO-d_6): δ 7.18 (d, J = 8.4 Hz, 2H), 6.53 (d, J = 8.4 Hz, 2H), 5.65 (br, 2H), 4.21 (d, J = 6.0 Hz, 2H); HRMS (ASAP): calcd for $C_{11}H_8N^+$ $[M-OH]^+$ 154.0657, found 154.0664.



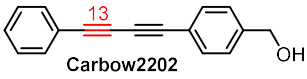
Carbow2237

^{1}H NMR (400 MHz; $CDCl_3$): δ 7.29 (d, J = 8.4 Hz, 2H), 6.59 (d, J = 8.4 Hz, 2H), 3.86 (br, 2H), 1.30 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 147.2, 134.1, 114.7, 111.3, 91.4, 72.2, 64.2, 53.5, 30.7, 28.4; HRMS (ASAP): calcd for $C_{14}H_{16}N^{+}$ $[M+H]^{+}$ 198.1283, found 198.1277.



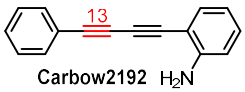
Carbow2215

^{1}H NMR (400 MHz; $CDCl_3$): δ 7.56-7.48 (m, 2H), 7.38-7.30 (m, 5H), 6.59 (d, J = 8.4 Hz, 2H), 3.89 (br, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 147.7, 134.2, 132.5, 128.9, 128.5, 122.3, 114.7, 110.7, 82.9, 80.9, 74.6, 72.2; HRMS (ASAP): calcd for $C_{16}H_{12}N^{+}$ $[M+H]^{+}$ 218.0970, found 218.0976.



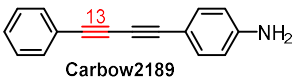
Carbow2202

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.60-7.45 (m, 4H), 7.43-7.27 (m, 5H), 4.72 (s, 2H); HRMS (ASAP): calcd for $^{12}C_{15}{}^{13}C_2H_{12}O$ $[M]^{+}$ 234.0955, found 234.0952.



Carbow2192

^{1}H NMR (400 MHz; $CDCl_3$): δ 7.58-7.49 (m, 2H), 7.40-7.31 (m, 4H), 7.19-7.13 (m, 1H), 6.72-6.66 (m, 2H), 4.32 (br, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 82.9 (d, J = 784.8 Hz), 73.9 (d, J = 784.8 Hz); HRMS (ASAP): calcd for $^{12}C_{14}{}^{13}C_2H_{12}N^{+}$ $[M+H]^{+}$ 220.1037, found 220.1035.

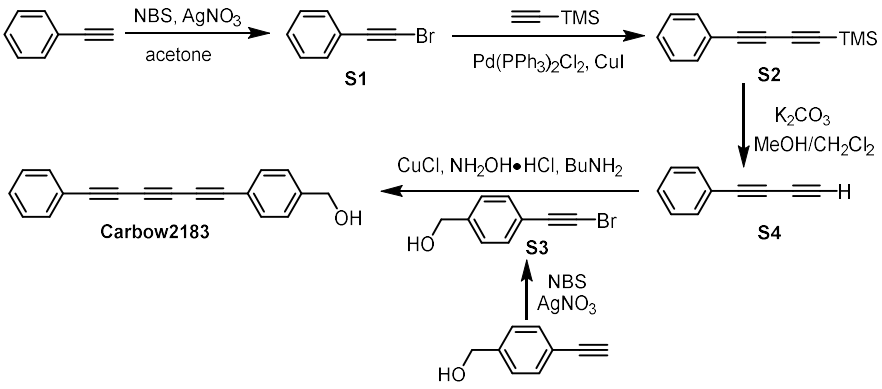


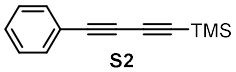
Carbow2189

^{1}H NMR (400 MHz; $CDCl_3$): δ 7.56-7.46 (m, 2H), 7.41-7.28 (m, 5H), 6.60 (d, J = 8.4 Hz, 2H), 3.89 (br, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 81.0 (d, J = 782.8 Hz), 74.4 (d, J = 782.8 Hz); HRMS (ASAP): calcd for $^{12}C_{14}{}^{13}C_2H_{12}N^{+}$ $[M+H]^{+}$ 220.1037, found 220.1038.

Synthesis of Carbow triynes

Carbow2183

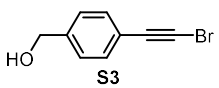




To a solution of phenylacetylene (1.32 mL, 12.0 mmol) in acetone (40 ml) was added *N*-bromosuccinimide (2.24 g, 12.6 mmol) and $AgNO_3$ (204 mg, 1.2 mmol). The reaction mixture was stirred 2 h at rt. Then, the mixture was filtered under reduced pressure, and the filtrate was poured into H_2O (50 mL). The product mixture was extracted with petroleum ether (3 × 30 mL). The combined organic layer was washed with brine and dried over $MgSO_4$, The solvent was concentrated under reduced pressure and the residue was purified by column chromatography to afford the phenylacetylene bromide product **S1** as a colorless liquid.

A mixture of the above phenylacetylene bromide **S1** (2.09 g, 11.6 mmol), CuI (66.4 mg, 0.35 mmol), and $Pd(PPh_3)_2Cl_2$ (0.24 g, 0.35 mmol) in a 100 mL three-neck flask was degassed and refilled with N_2. After this procedure was repeated three times, triethylamine (55 mL) and (trimethylsilyl)acetylene (2.45 mL, 17.4 mmol) were added with syringe. The reaction solution was stirred at 50 ^oC for 2 h, then the solvent was evaporated under reduced pressure, and the residue was further purified by column chromatography with petrol ether as the eluent to afford **S2** (1.47 g, 64%) as a yellow liquid.

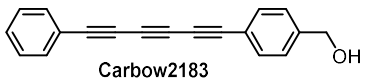
^1H NMR (400 MHz, $CDCl_3$): δ 7.52-7.48 (m, 2H), 7.40-7.30 (m, 3H), 0.25 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 132.8, 129.4, 128.5, 121.6, 90.8, 88.0, 76.9, 74.3, -0.2; HRMS (ASAP): calcd for $C_{13}H_{14}Si^+$ $[M]^+$ 198.0865, found 198.0873.



To a solution of 4-ethynylbenzyl alcohol (132 mg, 1.0 mmol) in acetone (8 ml) was added *N*-bromosuccinimide (187 mg, 1.05 mmol) and $AgNO_3$ (17.2 mg, 0.10 mmol). The reaction

mixture was stirred 3 h at rt. Then, the mixture was filtered under reduced pressure, and the filtrate was poured into H_2O (10 mL). The product mixture was extracted with ethyl ether (3 × 10 mL). The combined organic layer was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The resulting residue was purified by flash chromatography to obtain compound **S3** (177 mg, 84%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.43 (d, J = 8.0 Hz, 2H), 7.29 (d, J = 8.0 Hz, 2H), 4.67 (s, 2H), 1.92 (br, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 141.6, 132.3, 126.9, 122.0, 80.0, 64.9, 49.9; HRMS (ASAP): calcd for $C_9H_6Br^+$ $[M\text{-}OH]^+$ 192.9653, found 192.9658.

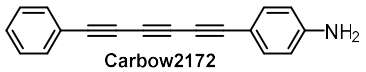


Carbow2183

To a solution of **S2** (30 mg, 0.15 mmol) in CH_2Cl_2-MeOH (1:1, 2 mL) was added K_2CO_3 (83 mg, 0.60 mmol). After the mixture was stirred at room temperature for 1 h, H_2O was added and the mixture was extracted with ether. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue **S4** was diluted with toluene (2 mL) and cooled to 0 °C. CuCl (5.2 mg, 0.05 mmol), NH_2OH • HCl (7.0 mg, 0.10 mmol) and $BuNH_2$ (60 μL, 0.6 mmol) were added in order. Alkynyl bromide **S3** (32 mg, 0.15 mmol) was diluted with 1 mL toluene and was added dropwise to the mixture. The reaction mixture was allowed to warm to room temperature and stirred for 10 h. The reaction was quenched with H_2O and extracted with ether. The organic layer was washed with H_2O and dried with $MgSO_4$. After the solvent was removed by rotovap, the product was purified by column chromatograph on silica to obtain **Carbow2183** (21 mg, 54%) as a yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58-7.48 (m, 4H), 7.43-7.37 (m, 1H), 7.37-7.30 (m, 4H), 4.72 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 142.6, 133.2, 133.0, 129.7, 128.5, 126.8, 121.0, 120.1, 78.6, 78.5, 74.5, 74.4, 66.5, 66.5, 64.8; HRMS (ASAP): calcd for $C_{19}H_{12}O^+$ $[M]^+$ 256.0888, found 256.0895.

The other **Carbow** triynes were prepared using the same procedures as **Carbow2183**.

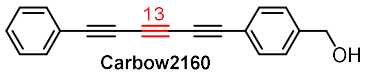


Carbow2172

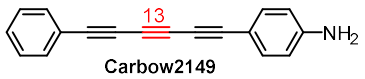
^{1}H NMR (400 MHz; $CDCl_3$): δ 7.55-7.50 (m, 2H), 7.40-7.30 (m, 5H), 6.58 (d, J = 8.4 Hz, 2H), 3.95 (br, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 148.1, 134.8, 133.0, 129.6, 128.6, 121.4, 114.7,

Nature Methods: doi:10.1038/nmeth.4578

109.7, 80.1, 78.4, 74.9, 72.9, 67.4, 65.9; HRMS (ASAP): calcd for $C_{18}H_{11}N^{+}$ $[M]^{+}$ 241.0891, found 241.0876.



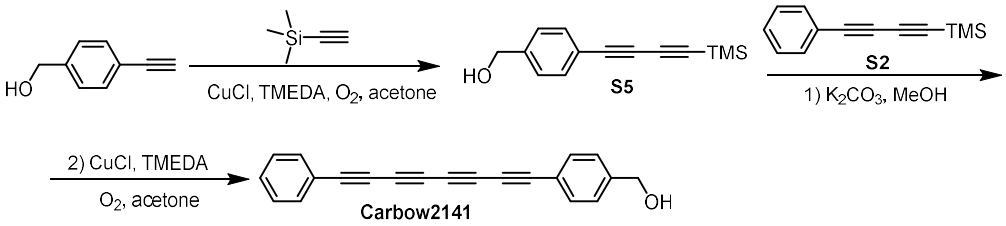
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.56-7.50 (m, 4H), 7.42-7.30 (m, 5H), 4.72 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 66.6; HRMS (ASAP): calcd for $^{12}C_{17}{}^{13}C_2H_{12}O$ $[M]^{+}$ 258.0955, found 258.0946.

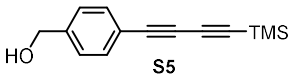


^{1}H NMR (400 MHz; $CDCl_3$): δ 7.56-7.50 (m, 2H), 7.41-7.29 (m, 5H), 6.58 (d, J = 8.4 Hz, 2H), 3.95 (br, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 66.8, 66.4; HRMS (ASAP): calcd for $^{12}C_{16}{}^{13}C_2H_{11}N^{+}$ $[M]^{+}$ 243.0959, found 243.0952.

Synthesis of Carbow tetraynes

Carbow2141

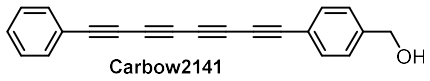




A solution of CuCl (120 mg, 1.2 mmol) and TMEDA (360 μL, 2.4 mmol) in acetone (7 ml) was bubbled with air for 10 mins at rt, then the mixture of (trimethylsilyl)acetylene (1.01 mL, 7.2 mmol) and 4-ethynylbenzyl alcohol (317 mg, 2.4 mmol) in CH_2Cl_2 were added and continued to stir with air at rt for 2 h, then concentrated at reduced pressure. The residue was subjected to chromatography to give compound **S5** (339 mg, 62%) as a yellow solid.

^{1}H NMR (400 MHz; $CDCl_3$): δ 7.47 (d, J = 8.0 Hz, 2H), 7.30 (d, J = 8.0 Hz, 2H), 4.67 (s, 2H), 1.95 (br, 1H), 0.23 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 142.3, 132.9, 126.9, 120.6, 90.8,

87.9, 76.7, 74.3, 64.8, -0.3; HRMS (ASAP): calcd for $C_{14}H_{15}Si^+$ $[M\text{-}OH]^+$ 211.0943, found 211.1019.



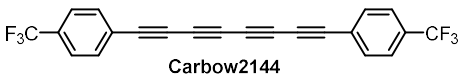
Carbow2141

To a solution of **S5** (100 mg, 0.44 mmol) and **S2** (130 mg, 0.66 mmol) in CH_2Cl_2-MeOH (1:1, 6 mL) was added K_2CO_3 (243 mg, 1.76 mmol). After the mixture was stirred at room temperature for 1 h, H_2O was added and the mixture was extracted with ether. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was used directly for next step.

A solution of CuCl (44 mg, 0.44 mmol) and TMEDA (132 μL, 0.88 mmol) in acetone (5 ml) was bubbled with air for 10 mins at rt, then the above mixture in CH_2Cl_2 were added and continued to stir with air at rt for 1 h, then concentrated at reduced pressure and purified by flash chromatography to obtain **Carbow2141** (64 mg, 52%) as a yellow solid.

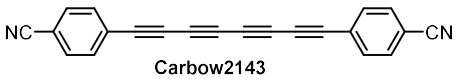
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56-7.51 (m, 4H), 7.44-7.38 (m, 1H), 7.37-7.31 (m, 4H), 4.72 (s, 2H), 1.78 (br, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 143.1, 133.5, 133.3, 130.1, 128.7, 126.9, 120.7, 119.8, 77.9, 77.7, 74.6, 74.5, 67.4, 67.3, 64.9, 63.9, 63.8; HRMS (ASAP): calcd for $C_{21}H_{12}O^+$ $[M]^+$ 280.0888, found 280.0876.

The other **Carbow** tetraynes were prepared using the same procedures as **Carbow2141**.



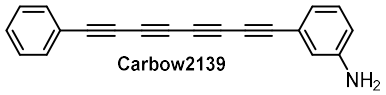
Carbow2144

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74-7.53 (m, 8H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 133.4, 131.6 (d, J = 32.8 Hz), 125.5 (q, J = 3.8 Hz), 124.3, 123.6 (d, J = 272.3 Hz), 76.3, 76.2, 68.0, 63.6; HRMS (ASAP): calcd for $C_{22}H_8F_6^+$ $[M]^+$ 386.0532, found 386.0542.

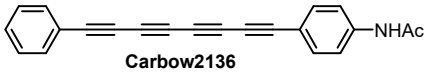


Carbow2143

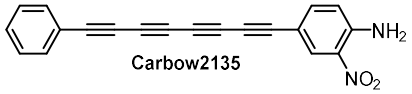
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72 (d, J = 8.0 Hz, 4H), 7.69 (d, J = 8.0 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 133.8, 132.5, 127.0, 118.1, 111.2, 78.3, 75.3, 68.7, 63.57; HRMS (ASAP): calcd for $C_{22}H_9N_2^+$ $[M+H]^+$ 301.0766, found 301.0757.



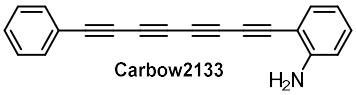
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58-7.50 (m, 2H), 7.45-7.38 (m, 1H), 7.38-7.31 (m, 2H), 7.12 (t, *J* = 7.9 Hz, 1H), 6.95 (d, *J* = 7.6 Hz, 1H), 6.83 (t, *J* = 1.9 Hz, 1H), 6.72 (dd, *J* = 8.0, 2.4 Hz, 1H), 3.74 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 146.4, 133.2, 130.0, 129.5, 128.6, 123.7, 121.1, 120.5, 118.8, 117.1, 78.1, 77.6, 74.5, 73.8, 67.3, 66.9, 63.8, 63.5; HRMS (ASAP): calcd for $C_{20}H_{12}N^+$ $[M+H]^+$ 266.0970, found 266.0974.



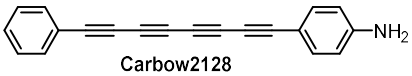
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57-7.52 (m, 2H), 7.51 (s, 3H), 7.43-7.38 (m, 1H), 7.38-7.32 (m, 2H), 7.21 (s, 1H), 2.20 (s, 3H); HRMS (ASAP): calcd for $C_{22}H_{14}NO^+$ $[M+H]^+$ 308.1075, found 308.1073.



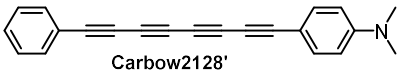
^{1}H NMR (400 MHz, $CDCl_3$): δ 8.36 (s, 1H), 7.54 (d, *J* = 7.5 Hz, 2H), 7.50-7.30 (m, 4H), 6.77 (d, *J* = 8.6 Hz, 1H), 6.35 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 145.2, 139.0, 133.2, 132.0, 130.0, 128.6, 120.5, 119.1, 108.9, 77.9, 76.1, 74.4, 74.1, 67.4, 67.2, 63.9, 63.6; HRMS (ASAP): calcd for $C_{20}H_{11}N_2O_2^+$ $[M+H]^+$ 311.0821, found 311.0815.



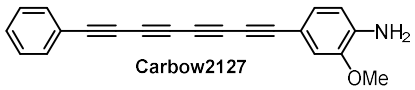
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.55 (d, *J* = 7.5 Hz, 2H), 7.47-7.29 (m, 4H), 7.19 (t, *J* = 7.7 Hz, 1H), 6.68 (t, *J* = 7.7 Hz, 2H), 4.36 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 150.9, 133.8, 133.2, 131.5, 130.0, 128.6, 120.6, 118.1, 114.5, 104.5, 79.7, 78.1, 75.2, 74.5, 68.8, 67.2, 64.6, 63.6; HRMS (ASAP): calcd for $C_{20}H_{12}N^+$ $[M+H]^+$ 266.0970, found 266.0972.



^{1}H NMR (400 MHz, $CDCl_3$): δ 7.60-7.47 (m, 2H), 7.45-7.28 (m, 5H), 6.58 (d, *J* = 8.4 Hz, 2H), 3.99 (br, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 148.3, 135.0, 133.1, 129.8, 128.5, 120.8, 114.6, 108.9, 79.3, 77.5, 74.6, 73.0, 67.6, 66.8, 64.5, 63.5; HRMS (ASAP): calcd for $C_{20}H_{12}N^+$ $[M+H]^+$ 266.0970, found 266.0974.

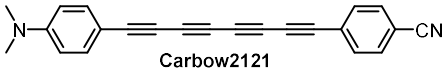


^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58-7.48 (m, 2H), 7.47-7.29 (m, 5H), 6.64-6.53 (m, 2H), 3.02 (s, 6H); HRMS (ASAP): calcd for $C_{22}H_{16}N^+$ $[M+H]^+$ 294.1283, found 294.1276.



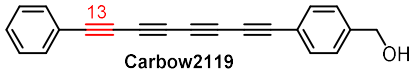
Carbow2127

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.53 (d, J = 7.4 Hz, 2H), 7.46-7.30 (m, 3H), 7.05 (d, J = 8.0 Hz, 1H), 6.92 (s, 1H), 6.59 (d, J = 8.1 Hz, 1H), 4.16 (s, 2H), 3.85 (s, 3H); HRMS (ASAP): calcd for $C_{21}H_{14}NO^+$ $[M+H]^+$ 296.1075, found 296.1071.



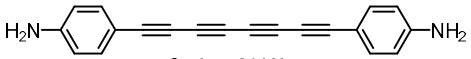
Carbow2121

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.65-7.59 (m, 4H), 7.44 (d, J = 8.4 Hz, 2H), 6.61 (d, J = 8.4 Hz, 2H), 3.04 (s, 6H); HRMS (ASAP): calcd for $C_{23}H_{15}N_2^+$ $[M+H]^+$ 319.1235, found 319.1239.



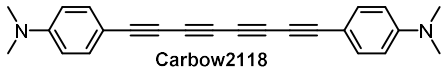
Carbow2119

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57-7.50 (m, 4H), 7.44-7.38 (m, 1H), 7.37-7.31 (m, 4H), 4.72 (s, 2H), 1.73 (br, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 78.1 (d, J = 786.0 Hz), 74.3 (d, J = 786.0 Hz); HRMS (ASAP): calcd for $^{12}C_{19}{}^{13}C_2H_{12}O$ $[M]^+$ 282.0955, found 282.0951.



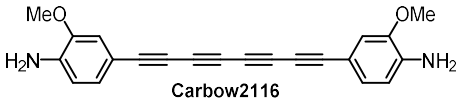
Carbow2119'

^{1}H NMR (500 MHz, MeOH-d_4): δ 7.20 (d, J = 8.5 Hz, 4H), 6.61 (d, J = 8.5 Hz, 4H); HRMS (ASAP): calcd for $C_{20}H_{13}N_2^+$ $[M+H]^+$ 281.1079, found 281.1080.



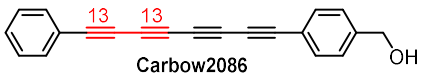
Carbow2118

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (d, J = 8.5 Hz, 4H), 6.58 (d, J = 8.5 Hz, 4H), 3.00 (s, 12H); HRMS (ASAP): calcd for $C_{24}H_{21}N_2^+$ $[M+H]^+$ 337.1705, found 337.1696.



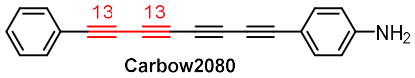
Carbow2116

^{1}H NMR (400 MHz, MeOH-d_4): δ 6.99 (dd, J = 8.1, 1.7 Hz, 2H), 6.94 (d, J = 1.7 Hz, 2H), 6.63 (d, J = 8.1 Hz, 2H), 3.84 (s, 6H); ^{13}C NMR (100 MHz, MeOH-d_4): δ 146.3, 140.7, 127.6, 113.9, 113.4, 106.8, 79.9, 71.4, 66.0, 63.8, 54.7; HRMS (ASAP): calcd for $C_{22}H_{17}N_2O_2^+$ $[M+H]^+$ 341.1290, found 341.1296.



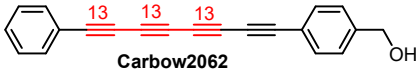
Carbow2086

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56-7.50 (m, 4H), 7.45-7.39 (m, 1H), 7.38-7.30 (m, 4H), 4.72 (s, 2H), 1.75 (br, 1H); HRMS (ASAP): calcd for $^{12}C_{17}{}^{13}C_4H_{11}$ $[M-OH]^+$ 267.0995, found 267.1011.



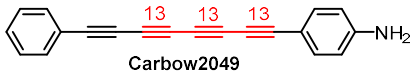
Carbow2080

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.61-7.47 (m, 2H), 7.45-7.29 (m, 5H), 6.65-6.51 (m, 2H), 3.98 (br, 2H); HRMS (ASAP): calcd for $^{12}C_{14}{}^{13}C_6H_{11}N^+$ $[M]^+$ 271.1093, found 271.1093.



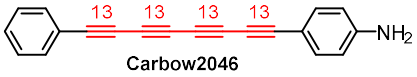
Carbow2062

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56-7.50 (m, 4H), 7.44-7.39 (m, 1H), 7.38-7.31 (m, 4H), 4.72 (s, 2H), 1.73 (br, 1H); HRMS (ASAP): calcd for $^{12}C_{15}{}^{13}C_6H_{11}$ $[M\text{-}OH]^+$ 269.1062, found 269.1116.



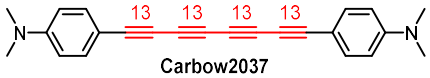
Carbow2049

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.53 (d, J = 8.0 Hz, 2H), 7.42-7.30 (m, 5H), 6.58 (d, J = 8.0 Hz, 2H), 3.97 (br, 2H); HRMS (ASAP): calcd for $^{12}C_{14}{}^{13}C_6H_{11}N^+$ $[M]^+$ 271.1093, found 271.1093.



Carbow2046

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.53 (d, J = 8.0 Hz, 2H), 7.42-7.32 (m, 5H), 6.58 (d, J = 8.0 Hz, 2H), 3.97 (br, 2H); HRMS (ASAP): calcd for $^{12}C_{12}{}^{13}C_8H_{12}N^+$ $[M+H]^+$ 274.1238, found 274.1222.

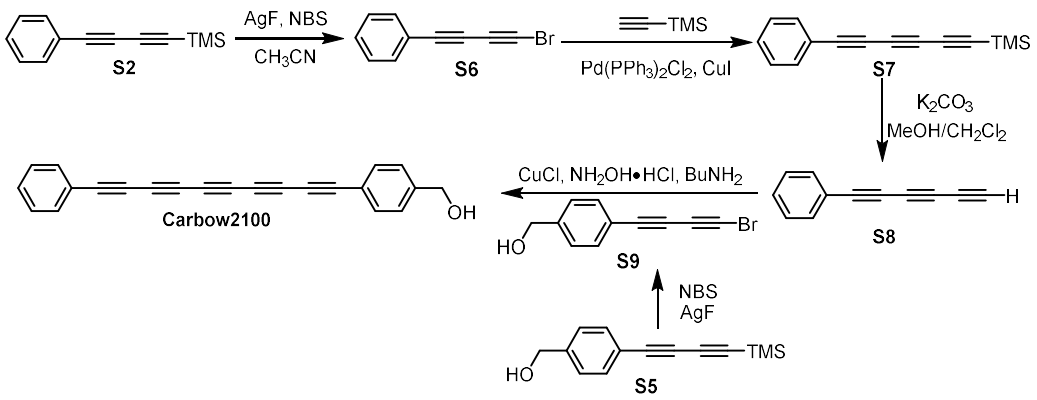


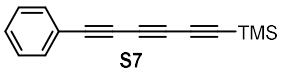
Carbow2037

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (dd, J = 8.8, 4.9 Hz, 4H), 6.59 (d, J = 8.8 Hz, 4H), 3.01 (s, 12H); HRMS (ASAP): calcd for $^{12}C_{16}{}^{13}C_8H_{21}N_2^+$ $[M+H]^+$ 345.1968, found 345.1971.

Synthesis of Carbow pentaynes

Carbow2100

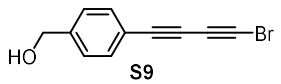




To a solution of **S2** (48 mg, 0.24 mmol) in acetonitrile with an addition of H_2O (18 μL, 1.0 mmol) was added AgF (30 mg, 0.24 mmol), and the mixture was stirred for 20 min. Next *N*-bromosuccinimide (51 mg, 0.28 mmol) was introduced, and the mixture was stirred for 3 h. Acetonitrile was removed under reduced pressure and the residue was diluted with ethyl ether. The organic layer was washed with H_2O and dried with $MgSO_4$. The solvent was removed and the residue was further purified by column chromatography to give the pure product **S6**.

A mixture of **S6**, CuI (4.4 mg, 0.024 mmol), and $Pd(PPh_3)_2Cl_2$ (16.8 mg, 0.024 mmol) in a three-neck flask was degassed and refilled with N_2. After this procedure was repeated three times, triethylamine (5 mL) and (trimethylsilyl)acetylene (47 mg, 0.48 mmol) were added with syringe. The reaction solution was stirred at 50 ^oC for 2 h, then the solvent was evaporated under reduced pressure, and the residue was further purified by column chromatography with petrol ether as the eluent to afford **S7** (36 mg, 67%) as a yellow liquid.

^1H NMR (400 MHz, $CDCl_3$): δ 7.54-7.49 (m, 2H), 7.42-7.36 (m, 1H), 7.36-7.29 (m, 2H), 0.23 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 133.1, 129.8, 128.5, 120.8, 89.0, 88.1, 76.9, 74.3, 66.8, 61.6, -0.5; HRMS (ASAP): calcd for $C_{15}H_{14}Si^+$ $[M]^+$ 222.0865, found 222.0843.

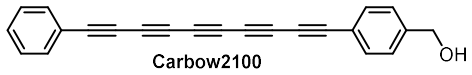


To a solution of **S5** (32 mg, 0.14 mmol) in acetonitrile with an addition of H_2O (18 μL, 1.0 mmol) was added AgF (18 mg, 0.14 mmol), and the mixture was stirred for 20 min. Next *N*-bromosuccinimide (29 mg, 0.16 mmol) was added, and the mixture was stirred for 5 h. Acetonitrile was removed under reduced pressure and the residue was diluted with ethyl ether.

Nature Methods: doi:10.1038/nmeth.4578

The organic layer was washed with H_2O, brine and dried with $MgSO_4$. The solvent was removed and the residue was further purified by column chromatography to give **S9** (24 mg, 73%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.49 (d, J = 8.3 Hz, 2H), 7.31 (d, J = 8.0 Hz, 2H), 4.69 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 142.4, 133.0, 126.8, 120.2, 74.3, 73.9, 65.4, 64.8, 44.5; HRMS (ASAP): calcd for $C_{11}H_6Br^+$ $[M\text{-}OH]^+$: 216.9653, found: 216.9661.

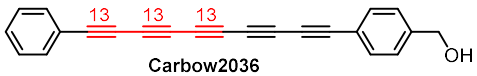


Carbow2100

To a solution of **S7** (11 mg, 0.05 mmol) in CH_2Cl_2-MeOH (1:1, 1 mL) was added K_2CO_3 (28 mg, 0.20 mmol). After the mixture was stirred at room temperature for 1 h, H_2O was added and the mixture was extracted with ether. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue **S8** was diluted with toluene (1 mL) and cooled to 0 °C. CuCl (5.2 mg, 0.05 mmol), $NH_2OH \cdot HCl$ (7.0 mg, 0.10 mmol) and $BuNH_2$ (60 μL, 0.6 mmol) were added in order. Alkynyl bromide 19 (12 mg, 0.05 mmol) in toluene (1 mL) was added dropwise to the mixture. The reaction mixture was allowed to warm to rt and stirred for 10 h. The reaction was quenched with H_2O and extracted with ether. The organic layer was washed with H_2O, brine and dried with $MgSO_4$. After the solvent was removed by rotovap, the product was purified by column chromatograph on silica to obtain **Carbow2100** (7.1 mg, 47%) as a yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.60-7.46 (m, 4H), 7.45-7.38 (m, 1H), 7.38-7.28 (m, 4H), 4.73 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 143.2, 133.6, 133.4, 130.2, 128.6, 126.8, 120.3, 119.3, 77.5, 74.4, 74.4, 67.3, 67.3, 64.7, 64.5, 64.5, 62.8, 62.8; HRMS (ASAP): calcd for $C_{23}H_{12}O^+$ $[M]^+$: 304.0888, found: 304.0864.

Carbow2036 was prepared using the same procedures as **Carbow2100**.

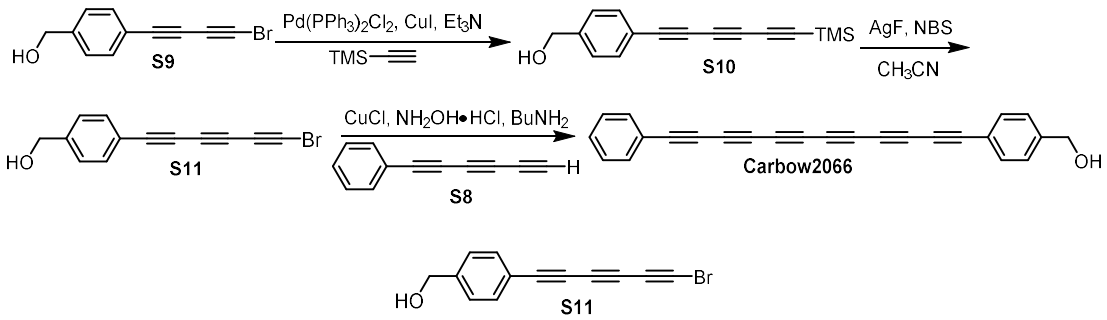


Carbow2036

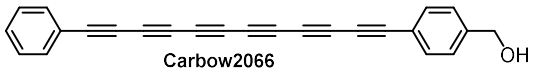
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58-7.47 (m, 4H), 7.45-7.37 (m, 1H), 7.36-7.29 (m, 4H), 4.72 (s, 2H); HRMS (ASAP): calcd for $^{12}C_{17}{}^{13}C_6H_{12}O$ $[M]^+$ 310.1089, found 310.1090.

Synthesis of Carbow hexaynes

Carbow2066



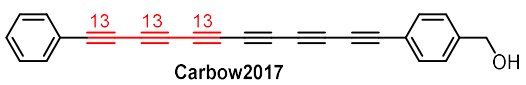
A mixture of the diacetylene bromide **S9** (29 mg, 0.12 mmol), CuI (2.2 mg, 0.012 mmol), and $Pd(PPh_3)_2Cl_2$ (8.4 mg, 0.012 mmol) in a three-neck flask was degassed and refilled with N_2. After this procedure was repeated three times, triethylamine (2 mL) and (trimethylsilyl)acetylene (24 mg, 0.24 mmol) were added with syringe. The reaction solution was stirred at 50 ^{o}C for 2 h, then the solvent was evaporated under reduced pressure, and the residue was further purified by column chromatography and the product **S10** was dissolved in acetonitrile (1 mL). H_2O (11 μL, 0.6 mmol) and AgF (15 mg, 0.12 mmol) were added and the mixture was stirred for 20 min. Next *N*-bromosuccinimide (27 mg, 0.15 mmol) was added, and the mixture was stirred for 5 h. Acetonitrile was removed under reduced pressure and the residue was diluted with ethyl ether. The organic layer was washed with H_2O, brine and dried with $MgSO_4$. The solvent was removed and the residue was further purified by column chromatography to give **S11** (16 mg, 52%).
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.51 (d, J = 7.9 Hz, 2H), 7.33 (d, J = 7.9 Hz, 2H), 4.71 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 142.9, 133.3, 126.8, 119.7, 75.6, 74.2, 67.2, 66.1, 64.7, 59.0, 42.9; HRMS (ASAP): calcd for $C_{13}H_6Br^+$ $[M\text{-}OH]^+$: 240.9653, found: 240.9647.



Compound **S8** was diluted with toluene (1 mL) and cooled to 0 °C. CuCl (5.2 mg, 0.05 mmol), NH_2OH • HCl (7.0 mg, 0.10 mmol) and $BuNH_2$ (60 μL, 0.6 mmol) were added in order. Alkynyl bromide **S11** (13 mg, 0.05 mmol) in toluene (1 mL) was added dropwise to the mixture. The reaction mixture was allowed to warm to rt and stirred for 10 h. The reaction was quenched with H_2O and extracted with ether. The organic layer was washed with H_2O, brine and dried with $MgSO_4$. After the solvent was removed by rotovap, the product was purified by column chromatograph on silica to obtain **Carbow2066** (6.8 mg, 42%) as a yellow solid.

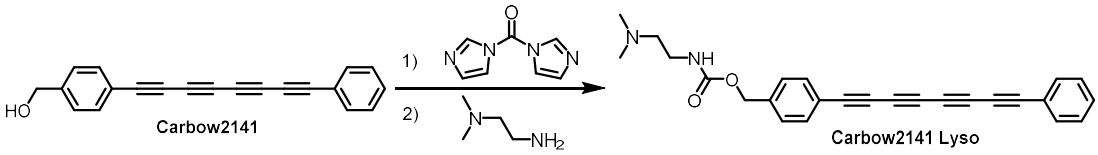
^{1}H NMR (400 MHz, DMSO-d_6): δ 7.67 (dd, J = 16.9, 7.6 Hz, 4H), 7.55 (t, J = 7.5 Hz, 1H), 7.45 (t, J = 7.6 Hz, 2H), 7.38 (d, J = 8.0 Hz, 2H), 4.52 (s, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 147.1, 134.1, 134.0, 131.9, 129.6, 127.2, 118.8, 116.7, 79.6, 79.2, 73.8, 73.5, 67.0, 66.9, 64.7, 64.6, 64.0, 63.9, 63.5, 63.3, 62.8; HRMS (ASAP): calcd for $C_{25}H_{13}O^+$ $[M+H]^+$: 329.0966, found: 329.0944.

Carbow2017 was prepared using the same procedures as **Carbow2066**.



^{1}H NMR (400 MHz, DMSO-d_6): δ7.68-7.59 (m, 4H), 7.57-7.50 (m, 1H), 7.48-7.35 (m, 4H), 4.50 (s, 2H); HRMS (ASAP): calcd for $^{12}C_{19}{}^{13}C_6H_{12}O$ $[M]^+$ 334.1089, found 334.1080.

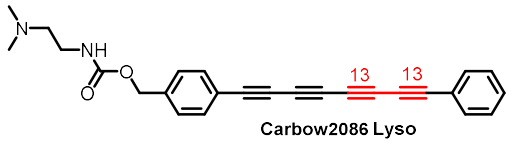
Synthesis of Carbow Lyso



To **Carbow2141** (8.4 mg, 0.03 mmol) in CH_2Cl_2 (1 ml) was added 1,1'-carbonyldiimidazole (9.7 mg, 0.06 mmol) at rt. After the mixture was stirred at rt for 6 h, H_2O was added and the mixture was extracted with CH_2Cl_2. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (1 ml) and *N,N*-dimethylethylenediamine (6.6 µL, 0.06 mmol) was added and continued to stir at rt for 20 h, then concentrated at reduced pressure and purified by flash chromatography to obtain **Carbow2141 Lyso** as a yellow solid (9.8 mg, 82%).

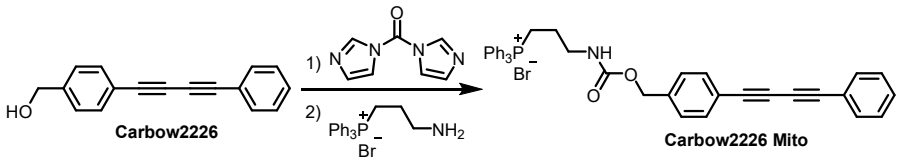
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.61-7.47 (m, 4H), 7.43-7.28 (m, 5H), 5.54 (br, 1H), 5.09 (s, 2H), 3.35-3.25 (m, 2H), 2.49 (t, J = 5.6 Hz, 2H), 2.28 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.3, 139.0, 133.4, 133.3, 130.1, 128.7, 128.0, 120.6, 120.2, 77.8, 77.5, 74.8, 74.5, 67.4, 67.3, 65.9, 63.8, 63.7, 58.2, 45.1, 38.2; HRMS (ESI): calcd for $C_{26}H_{23}N_2O_2^+$ $[M+H]^+$: 399.1894, found: 399.1894.

Carbow2086 Lyso



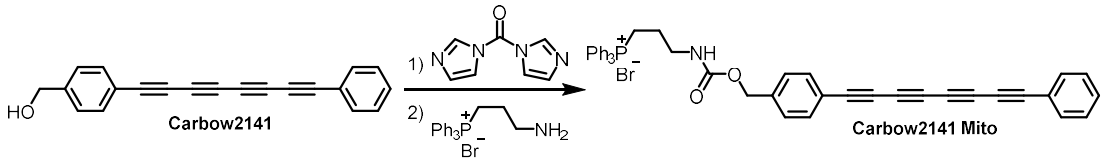
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56-7.49 (m, 4H), 7.43-7.38 (m, 1H), 7.36-7.31 (m, 4H), 5.66 (br, 1H), 5.10 (s, 2H), 3.35-3.27 (m, 2H), 2.57 (t, J = 5.6 Hz, 2H), 2.35 (s, 6H); HRMS (ESI): calcd for $^{12}C_{22}{}^{13}C_4H_{23}N_2O_2^+$ $[M+H]^+$: 395.1760, found: 395.1767.

Synthesis of Carbow Mito



To **Carbow2226** (10.5 mg, 0.045 mmol) in CH_2Cl_2 (1 ml) was added 1,1'-carbonyldiimidazole (11.2 mg, 0.068 mmol) at rt. After the mixture was stirred at room temperature for 3 h, H_2O was added and the mixture was extracted with CH_2Cl_2. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (1 ml) and (3-aminopropyl) triphenylphosphonium bromide (43 mg, 0.09 mmol) was added and continued to stir at rt for 15 h, then concentrated at reduced pressure and purified by flash chromatography to obtain **Carbow2226 Mito** as a yellow solid (23 mg, 78%).

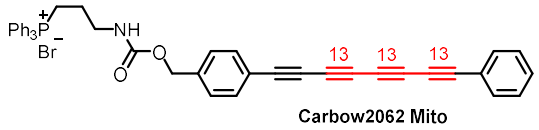
^{1}H NMR (400 MHz; $CDCl_3$): δ 7.79-7.69 (m, 8H), 7.67-7.60 (m, 6H), 7.53-7.46 (m, 3H), 7.43-7.38 (m, 2H), 7.37-7.28 (m, 5H), 5.05 (s, 2H), 3.81-3.69 (m, 2H), 3.50-3.40 (m, 2H), 1.88-1.76 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.9, 138.8, 135.2 (d, J = 12.0 Hz), 133.6, 133.5, 132.6, 130.6, 129.3, 128.5, 127.7, 121.8, 120.9, 117.8, 81.7, 81.6, 74.1, 74.0, 65.5, 40.4 (d, J = 68.8 Hz), 22.8 (d, J = 15.2 Hz), 20.5; HRMS (ESI): calcd for $C_{39}H_{33}NO_2P^+$ $[M\text{-}Br]^+$: 578.2249, found: 578.2249.



To **Carbow2141** (8.4 mg, 0.03 mmol) in CH_2Cl_2 (1 ml) was added 1,1'-carbonyldiimidazole (9.7 mg, 0.06 mmol) at rt. After the mixture was stirred at room temperature for 6 h, H_2O was added and the mixture was extracted with CH_2Cl_2. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (1 ml) and (3-aminopropyl) triphenylphosphonium bromide (20 mg, 0.05 mmol) was added and continued to stir at rt for 20 h, then concentrated at reduced pressure and purified by flash chromatography to obtain **Carbow2141 Mito** as a yellow solid (16 mg, 75%).

^{1}H NMR (400 MHz; $CDCl_3$): δ 7.80-7.60 (m, 14H), 7.57-7.49 (m, 3H), 7.44-7.28 (m, 7H), 5.05 (s, 2H), 3.78-3.65 (m, 2H), 3.50-3.40 (m, 2H), 1.90-1.75 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.9, 139.8, 135.2 (d, J = 12.0 Hz), 133.6, 133.5, 133.3 (d, J = 12.0 Hz), 130.6, 130.1, 128.6, 127.7, 120.6, 119.6, 117.8, 77.9, 77.8, 74.5, 67.4, 67.3, 65.5, 63.8, 40.5 (d, J = 68.8 Hz), 22.8 (d, J = 15.2 Hz), 20.3; HRMS (ESI): calcd for $C_{43}H_{33}NO_2P^+$ $[M\text{-}Br]^+$: 626.2249, found: 626.2252.

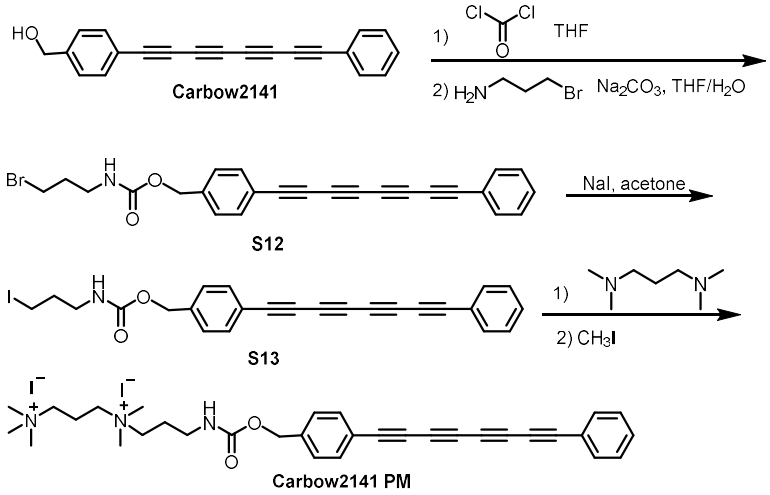
Carbow2062 Mito



Carbow2062 Mito

^{1}H NMR (400 MHz; $CDCl_3$): δ 7.80-7.75 (m, 3H), 7.73-7.62 (m, 11H), 7.57-7.50 (m, 3H), 7.46-7.30 (m, 7H), 5.06 (s, 2H), 3.70-3.60 (m, 2H), 3.49-3.40 (m, 2H), 1.88-1.78 (m, 2H); HRMS (ESI): calcd for $^{12}C_{37}{}^{13}C_6H_{33}NO_2P^+$ $[M\text{-}Br]^+$: 632.2450, found: 632.2452.

Synthesis of Carbow2141 PM



Carbow2141 (168 mg, 0.6 mmol) was dissolved in THF (4 mL). The resulting solution was then added dropwise into a phosgene solution (0.90 mL, 1.2 mmol) at rt and was stirred for 24 h. The residual phosgene and solvent were then removed by high vacuum to produce a yellowish solid and it was dissolved in THF (2 mL) which was directly used for the subsequent reaction without further purification.

To a mixture of 3-bromopropan-1-amine hydrobromide (263 mg, 1.2 mmol) and sodium carbonate (254 mg, 2.4 mmol) in THF (4 mL) and water (3 mL) was added dropwise the above chloroformate at rt and then the reaction mixture was allowed to stir at rt for 1 h. The reaction mixture was diluted with ethyl ether then the organic layer was washed with brine and dried with $MgSO_4$. After the solvent was removed by rotovap, the product was purified by column chromatograph on silica to obtain **S12** (178 mg, 67%) as a yellow solid.

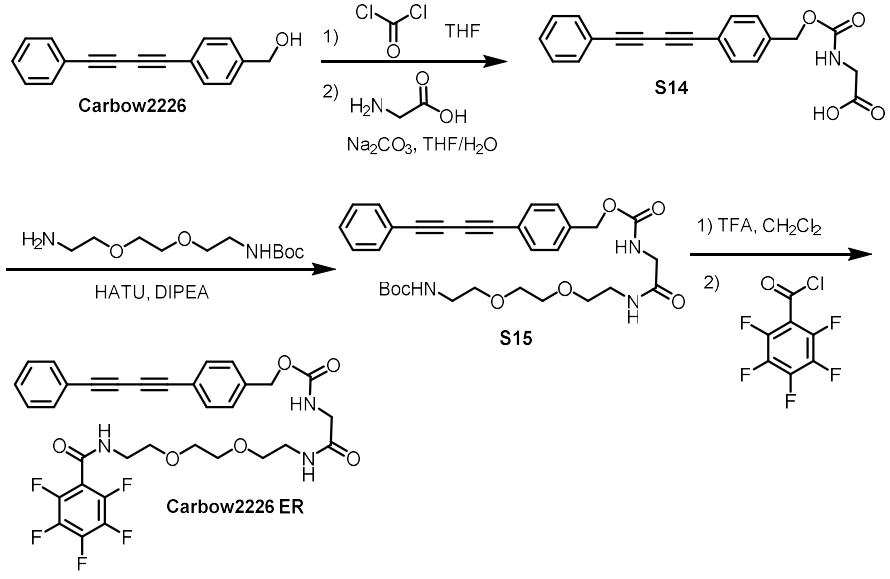
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57-7.49 (m, 4H), 7.43-7.38 (m, 1H), 7.37-7.28 (m, 4H), 5.09 (s, 2H), 4.92 (br, 1H), 3.43 (t, J = 6.4 Hz, 2H), 3.36 (q, J = 6.4 Hz, 2H), 2.13-2.03 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.2, 138.7, 133.5, 133.3, 130.2, 128.7, 128.1, 120.6, 120.4, 77.9,

74.9, 74.5, 67.5, 67.3, 66.1, 63.9, 63.7, 39.6, 32.5, 30.6; HRMS (ASAP): calcd for $C_{25}H_{18}BrNO_2^+$ $[M]^+$ 443.0521, found 443.0533.

To a solution of **S12** (22 mg, 0.05 mmol) in acetone (1 mL) was added NaI (75 mg, 0.50 mmol). After the mixture was stirred at 60 ^oC for 12 h, H_2O was added and the mixture was extracted with ether. The organic phase was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue **S13** was dissolved in THF (1 mL) and *N,N,N′,N′*-tetramethyl-1,3-propanediamine (42 μL, 0.25 mmol) was added. The mixture was stirred at 50 ^oC for 18 h. The solvent was removed by evaporation under reduced pressure and the crude product was washed several times with diethyl ether. This mono-charged intermediate was dissolved in CH_3CN (1 mL) and CH_3I (62 μL, 1 mmol) was added to the solution. After stirring the reaction mixture at 60 ^oC for 16 h, the solvent and CH_3I was evaporated under reduced pressure to give a yellow solid **Carbow2141 PM** (16 mg, 43%).

^1H NMR (400 MHz, CD_3OD): δ 7.60-7.54 (m, 4H), 7.50-7.45 (m, 1H), 7.44-7.36 (m, 4H), 5.12 (s, 2H), 4.51 (s, 1H), 3.57-3.44 (m, 6H), 3.30-3.28 (m, 2H), 3.27 (s, 9H), 3.21 (s, 6H), 2.45-2.34 (m, 2H), 2.10-2.00 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD): δ 158.6, 141.1, 134.4, 134.2, 131.6, 129.9, 128.9, 121.3, 120.8, 79.0, 78.6, 74.6, 74.3, 67.5, 67.2, 66.8, 64.3, 64.1, 63.7, 61.4, 54.8, 54.3, 51.9, 38.8, 24.5, 19.0, 15.4; HRMS (ESI): calcd for $C_{33}H_{39}N_3O_2^{2+}$ $[M-2I]^{2+}$ 254.6515, found 254.6528.

Synthesis of Carbow2226 ER



To a solution of **Carbow2226** (70 mg, 0.3 mmol) in THF (3 mL) was added a phosgene solution (0.63 mL, 0.9 mmol) at rt and was stirred for 24 h. The residual phosgene and solvent were then removed by high vacuum to produce a solid and it was dissolved in THF (2 mL) which was directly used for the subsequent reaction without further purification.

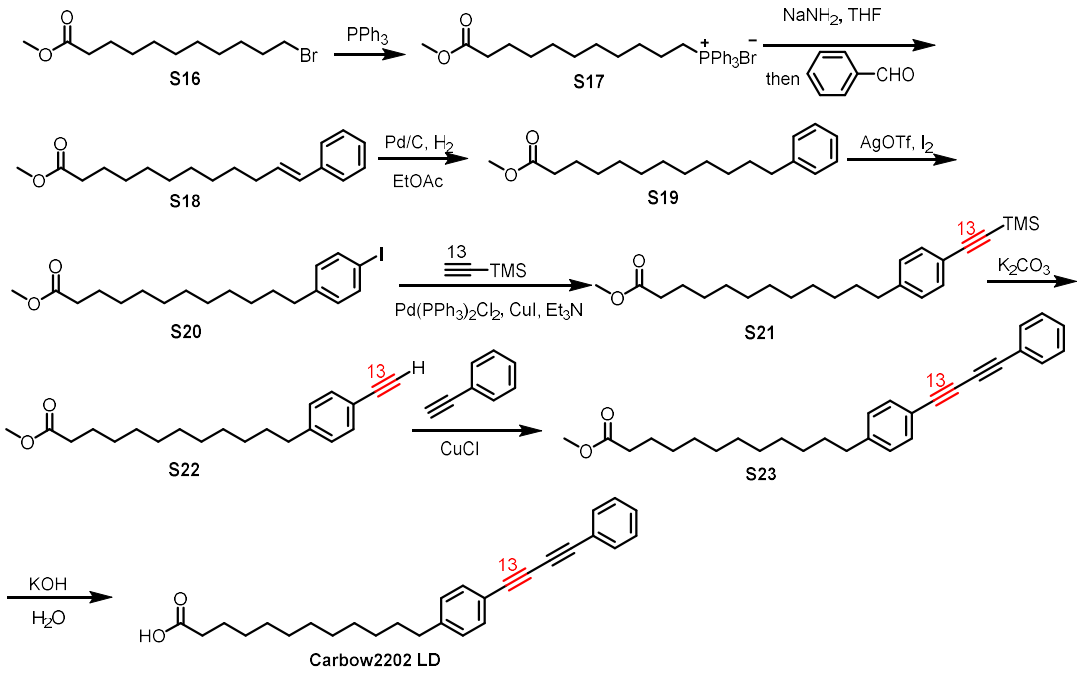
To a mixture of glycine (68 mg, 0.9 mmol) and sodium carbonate (159 mg, 1.5 mol) in THF (2 mL) and water (1 mL) was added dropwise the above chloroformate at rt and then the reaction mixture was allowed to stir at rt for 2 h. The reaction mixture was quenched with 0.1 M HCl and diluted with ethyl ether. The organic layer was washed with brine and dried with $MgSO_4$. After the solvent was removed by rotovap, the product was purified by column chromatograph on silica to obtain compound **S14** (58 mg, 58%) as a white solid.

^{1}H NMR (400 MHz, CD_3OD): δ 7.55-7.50 (m, 4H), 7.43-7.35 (m, 5H), 5.13 (s, 2H), 3.84 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD): δ 173.5, 158.9, 139.9, 133.5, 133.4, 130.5, 129.7, 128.8, 122.9, 122.4, 82.3, 81.9, 74.5, 74.3, 67.0, 43.1; HRMS (ASAP): calcd for $C_{20}H_{14}NO_4^-$ $[M-H]^-$ 332.0923, found 332.0931.

S14 (6.8 mg, 0.02 mmol) in DMF (1 mL) was treated with DIPEA (18 μL, 0.1 mmol) and HATU (7.6 mg, 0.02 mmol) at rt. After 5 min, *N*-Boc-2,2'-(ethylenedioxy)-diethyl-amine (7.4 mg, 0.03 mmol) was added. The mixture was stirred overnight at rt and the reaction was quenched by adding water. The product was extracted with DCM and dried with $MgSO_4$. The solvent was concentrated on a rotary evaporator and the residue was purified on a silica gel column to give the coupling product **S15**, which was dissolved in TFA-CH_2Cl_2 (1:4, 1 mL). The reaction mixture was stirred for 1 h at rt and was concentrated to yield the amine product without further purification. The amine was dissolved in CH_2Cl_2 (1 mL) and cooled to 0 ^{o}C. Triethylamine (28 μL, 0.2 mmol) and pentafluorobenzoyl chloride (6 μL, 0.04 mmol) was added. After the mixture was stirred at 0 ^{o}C for 1 h, H_2O was added and the mixture was extracted with CH_2Cl_2. The organic phase was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was further purified by column chromatography to afford **Carbow2226 ER** (6.3 mg, 48%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.54-7.48 (m, 4H), 7.40-7.27 (m, 5H), 6.97 (br, 1H), 6.48 (br, 1H), 5.61 (br, 1H), 5.08 (s, 2H), 3.81 (d, J = 5.8 Hz, 2H), 3.68-3.58 (m, 8H), 3.56-3.50 (m, 2H), 3.45-3.40 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.0, 157.8, 156.6, 137.4, 132.8, 132.6, 129.4, 128.6, 128.0, 121.9, 121.8, 82.0, 81.1, 74.5, 73.9, 70.5, 70.4, 69.6, 69.5, 66.6, 44.6, 40.3, 39.3; HRMS (APCI): calcd for $C_{33}H_{28}F_5N_3O_6Na^+$ $[M+Na]^+$ 680.1796, found 680.1791.

Synthesis of Carbow2202 LD



To a solution of methyl 11-bromoundecanoate **S16** (852 mg, 2.9 mmol) in CH_3CN (10 mL) was added PPh_3 (836 mg, 3.2 mmol). After the mixture was stirred at 85 °C for 48 h, the solvent was removed by evaporation under reduced pressure and the residue was washed three times with diethyl ether. The crude product **S17** was dissolved in THF (10 mL) and $NaNH_2$ (170 mg, 4.3 mmol) was added at 0 °C. After stirring the reaction mixture at 0 °C for 30 min, benzaldehyde (0.35 mL, 3.5 mmol) was added and it was allowed to warm to rt and continued to stir at rt for 10h. H_2O was added dropwise to quench the reaction and the mixture was extracted with Et_2O, then the combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column chromatography to afford **S18** (468 mg, 56%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38-7.20 (m, 5H), 6.45 (d, J = 11.6 Hz, 1H), 5.70 (dt, J = 11.8, 7.2 Hz, 1H), 3.70 (s, 3H), 2.41-2.30 (m, 4H), 1.75-1.60 (m, 2H), 1.52-1.43 (m, 2H), 1.34-1.20 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.4, 137.9, 133.3, 128.9, 128.2, 126.5, 125.8, 51.5, 34.2, 30.0, 29.6, 29.5, 29.4, 29.3, 29.2, 28.7, 25.0; HRMS (APCI): calcd for $C_{19}H_{29}O_2^+$ $[M+H]^+$ 289.2168, found 289.2167.

To a solution of **S18** (173 mg, 0.6 mmol) in EtOAc (4 mL), 10% Pd/C catalyst (10 mg) was added. The reaction mixture was stirred under H_2 atmosphere overnight at rt. The catalyst was removed by filtration through a pad of Celite, and the organic solvent was evaporated under reduced pressure. The crude product **S19** was dissolved in CH_2Cl_2 (5 mL) then AgOTf (154 mg, 0.6 mmol) and iodine (152 mg, 0.6 mmol) were added at rt. After stirring the reaction mixture at rt for 2 h, saturated sodium bisulfite solution was added and stirred at rt for 10 min. The mixture

was extracted with ether and the organic layer was washed with H_2O, brine and dried with $MgSO_4$. The solvent was removed under reduced pressure and the residue was further purified by column chromatography to afford **S20** (187 mg, 75%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.60 (d, J = 8.0 Hz, 2H), 6.94 (d, J = 8.0 Hz, 2H), 3.69 (s, 3H), 2.56 (t, J = 7.2 Hz, 2H), 2.33 (t, J = 7.2 Hz, 2H), 1.69-1.54 (m, 4H), 1.40-1.23 (m, 14H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.3, 142.5, 137.3, 130.6, 90.6, 51.5, 35.5, 34.2, 31.3, 29.6, 29.5, 29.4, 29.3, 29.2, 25.0; HRMS (ASAP): calcd for $C_{19}H_{30}IO_2^+$ $[M+H]^+$ 417.1290, found 417.1298.

A mixture of aryl iodide **S20** (166 mg, 0.4 mmol), CuI (7.6 mg, 0.04 mmol), and $Pd(PPh_3)_2Cl_2$ (28 mg, 0.04 mmol) in a 25 mL three-neck flask was degassed and refilled with N_2. After this procedure was repeated three times, triethylamine (5 mL) and (trimethylsilyl)acetylene-1,2-$^{13}C_2$ (60 mg, 0.6 mmol) were added with syringe. The reaction solution was stirred at 40 ^{o}C for 2 h, then the solvent was evaporated under reduced pressure. The residue was further purified by column chromatography to give the pure product **S21** which was dissolved in MeOH (4 mL) and K_2CO_3 (138 mg, 1.0 mmol) was added. After the mixture was stirred at room temperature for 3 h, H_2O was added and the mixture was extracted with ether. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography to afford **S22** (91 mg, 72%).

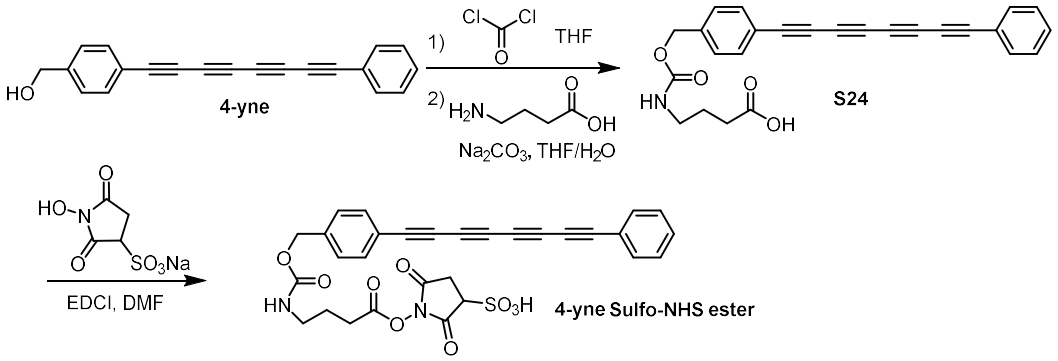
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40 (dd, J = 5.4, 7.8 Hz, 2H), 7.13 (d, J = 7.8 Hz, 2H), 3.67 (s, 3H), 3.03 (dd, J = 52.2, 248.4 Hz, 1H), 2.60 (t, J = 7.6 Hz, 2H), 2.30 (t, J = 7.6 Hz, 2H), 1.65-1.54 (m, 4H), 1.35-1.24 (m, 14H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.4, 144.1, 132.1 (t, J = 8.4 Hz), 128.5 (d, J = 21.4 Hz), 119.7 (d, J = 55.2 Hz), 84.0 (d, J = 698.2 Hz), 76.4 (d, J = 698.2 Hz), 51.5, 36.0, 34.2, 31.3, 29.6, 29.5, 29.4, 29.3, 29.2, 25.1; HRMS (ASAP): calcd for $^{12}C_{19}{}^{13}C_2H_{31}O_2^+$ $[M+H]^+$ 317.2392, found 317.2401.

A solution of CuCl (5.0 mg, 0.05 mmol) and TMEDA (15 μL, 0.10 mmol) in acetone (1 ml) was bubbled with air for 10 mins at rt, then the mixture of **S22** (16 mg, 0.05 mmol) and phenylacetylene (22 μl, 0.2 mmol) in CH_2Cl_2 (1 ml) were added and continued to stir with air at rt for 1 h. The solvent was evaporated at reduced pressure and the residue was further purified by column chromatography to give the pure **S23**, which was dissolved in THF-MeOH (2:1, 0.9 mL) and 3.0 M KOH (0.1 mL, 0.3 mmol) was added. After the mixture was stirred at room temperature for 1 h, 1.0 M HCl was added and the mixture was extracted with ether. The organic phase was dried over Na_2SO_4 and the solvent was evaporated. The residue was purified by column chromatography to afford **Carbow2202 LD** (11.6 mg, 55%).

^{1}H NMR (400 MHz; $CDCl_3$): δ 7.56-7.49 (m, 2H), 7.47-7.41 (m, 2H), 7.39-7.30 (m, 3H), 7.15 (d, J = 8.0 Hz, 2H), 2.60 (t, J = 7.6 Hz, 2H), 2.35 (t, J = 7.6 Hz, 2H), 1.69-1.53 (m, 4H), 1.37-1.23

(m, 14H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.6, 144.8, 132.6, 132.5, 129.2, 128.7 (d, J = 22.6 Hz), 128.5, 122.1 (d, J = 15.6 Hz), 119.0 (dd, J = 60.0, 364.0 Hz), 82.1 (d, J = 784.0 Hz), 73.3 (d, J = 784.0 Hz), 36.1, 34.1, 31.3, 29.6, 29.5, 29.4, 29.3, 29.2, 24.8; HRMS (ASAP): calcd for $^{12}C_{26}{}^{13}C_2H_{31}O_2^-$ [M-H]$^-$ 401.2391, found 401.2402.

Synthesis of 4-yne Sulfo-NHS ester



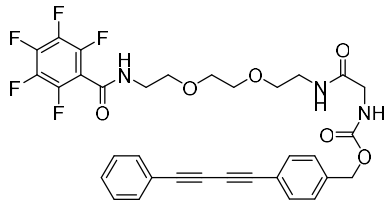
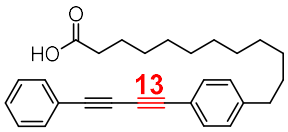
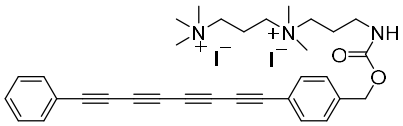
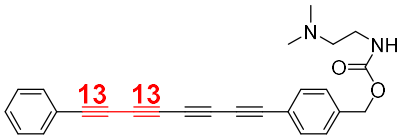
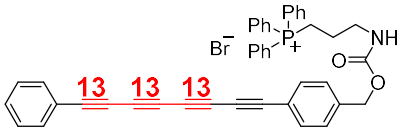
4-yne (8.4 mg, 0.03 mmol) was dissolved in THF (1 mL). The resulting solution was then added dropwise into a phosgene solution (0.045 mL, 0.06 mmol) at rt and was stirred for 24 h. The residual phosgene and solvent were then removed by high vacuum to produce a yellowish solid and it was dissolved in THF (1 mL) which was directly used for the subsequent reaction without further purification.

To a mixture of 4-Aminobutanoic acid (6.2 mg, 0.06 mmol) and sodium carbonate (10.5 mg, 0.1 mol) in THF (1 mL) and water (1 mL) was added dropwise the above chloroformate at rt and then the reaction mixture was allowed to stir at rt for 1 h. The reaction mixture was quenched with 0.1 M HCl and diluted with ethyl acetate. The organic layer was washed with brine and dried with $MgSO_4$. After the solvent was removed by rotovap, the product **S24** in DMF (1 mL) were added *N*-hydroxysulfosuccinimide sodium salt (21.7 mg, 0.10 mmol) and EDCI (19.2 mg, 0.10 mmol). After the mixture was stirred at rt for 48 h, H_2O was added and the mixture was purified by RP-HPLC to afford **4-yne Sulfo-NHS ester** (4.6 mg, 26%).

^1H NMR (500 MHz, DMF-d_7): δ 7.75-7.68 (m, 4H), 7.59-7.54 (m, 1H), 7.52-7.44 (m, 4H), 7.40 (t, J = 5.0 Hz, 1H), 5.14 (s, 2H), 4.04 (dd, J = 2.0, 8.5 Hz, 1H), 3.30-3.18 (m, 3H), 3.12-3.05 (m, 1H), 2.75 (t, J = 7.2 Hz, 2H), 1.94-1.86 (m, 2H); ^{13}C NMR (125 MHz, DMF-d_7): δ 169.6, 166.3, 156.9, 141.6, 134.1, 134.0, 131.6, 129.7, 128.4, 120.0, 119.1, 79.3, 79.1, 73.9, 73.8, 67.0, 65.4, 64.1, 57.5, 40.3, 31.9, 28.5, 25.6; HRMS (ASAP): calcd for $C_{30}H_{21}N_2O_9S^-$ [M-H]$^-$: 585.0968, found: 585.0976.

Supplementary Tables

Supplementary Table 1. Probes for 10-color organelle imaging in live cells

Carbow probes	Chemical structures	Fluorescent reporters
Carbow2226 ER		**NucBlue**
Carbow2202 LD		**Actin-GFP**
Carbow2141 PM		**BODIPY TR ceramide**
Carbow2086 Lyso		**SiR-Tubulin**
Carbow2062 Mito		**FM 4-64**

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Supplementary Table 2. Recipe of 10-frequency barcoding for SRS measurement

Barcodes	Carbow 2017 (μM)	Carbow 2036 (μM)	Carbow 2066 (μM)	Carbow 2100 (μM)	Carbow 2119 (μM)	Carbow 2141 (μM)	Carbow 2160 (μM)	Carbow 2183 (μM)	Carbow 2202 (μM)	Carbow 2226 (μM)
1111111111	7.3	7.2	3.4	3.0	12.8	4.0	23.6	9.2	25.5	42.5
1212121212	7.9	15.0	3.6	8.1	14.4	11.0	22.7	20.9	13.2	85.7
2101221012	14.3	7.0	0.0	2.8	25.3	7.8	22.3	0.0	34.4	83.6
0121001210	0.0	7.2	7.0	2.9	0.0	0.0	23.1	18.6	15.4	0.0

Supplementary Table 3. Recipe of 5-frequency barcoding for spontaneous Raman measurement

Barcodes	Carbow2141 (μM)	Carbow2160 (μM)	Carbow2183 (μM)	Carbow2202 (μM)	Carbow2226 (μM)
11111	15.3	57.7	31.2	51.9	93.2
22222	29.4	111.1	60.2	100.0	179.5
01210	0.0	59.1	64.0	26.6	0.0
12121	16.6	113.6	30.8	127.8	91.9
21112	32.7	56.1	30.4	50.5	181.5
12221	16.6	113.6	61.5	102.2	91.8
21012	32.7	56.1	0.0	84.2	181.4
21212	29.8	56.3	61.0	25.3	181.9
22022	23.6	111.6	0.0	167.5	180.5
22122	29.4	111.1	30.1	125.0	179.6

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